



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/31, 15/80, C07K 14/375, C12N 1/15, 1/21, A23L 1/00, A01N 63/00	A1	(11) International Publication Number: WO 96/41882 (43) International Publication Date: 27 December 1996 (27.12.96)
(21) International Application Number: PCT/NL96/00234 (22) International Filing Date: 11 June 1996 (11.06.96) (30) Priority Data: 95201555.0 12 June 1995 (12.06.95) EP <i>(34) Countries for which the regional or international application was filed:</i> NL et al. 95201556.8 12 June 1995 (12.06.95) EP <i>(34) Countries for which the regional or international application was filed:</i> NL et al. 95201558.4 12 June 1995 (12.06.95) EP <i>(34) Countries for which the regional or international application was filed:</i> NL et al. (71) Applicants (for all designated States except US): PROEFSTATION VOOR DE CHAMPIGNONCULTUUR [NL/NL]; P.O. Box 6042, NL-5960 AA Horst (NL). RIJKS LANDBOUWUNIVERSITEIT WAGENINGEN [NL/NL]; Costerweg 50, NL-6701 BH Wageningen (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): VAN GRIENSVEN, Leonardus, Johannes, Lambertus, Donatus [NL/NL]; Deltasingel 15, NL-5751 SL Deurne (NL). DE GROOT, Petrus, Wilhelmus, Johannes [NL/NL]; Groen van Prin-		sterestraat 111, NL-6702 CR Wageningen (NL). SCHAAAP, Petrus, Johannes [NL/NL]; Karveel 30, NL-1625 EP Hoor (NL). VISSER, Jacob [NL/NL]; Hinkeloordseweg 5, NL-6703 CK Wageningen (NL). (74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL). (81) Designated States: AU, BR, CA, CN, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: HYDROPHOBINS FROM EDIBLE FUNGI, GENES, NUCLEOTIDE SEQUENCES AND DNA-FRAGMENTS ENCODING FOR SAID HYDROPHOBINS, AND EXPRESSION THEREOF		
(57) Abstract <p>The present invention relates to a hydrophobin, or a protein or polypeptide essentially corresponding thereto, derived from an edible fungus, preferably with GRAS status, more preferably from the genus <i>Agaricus</i>, such as <i>Agaricus bisporus</i>. The invention further relates to genes, nucleotide sequences or DNA fragments encoding a ripening form of such a hydrophobin, as well as recombinant DNA material containing said gene, nucleotide sequence or DNA fragment. The invention further relates to a process for producing a ripening form of a hydrophobin, a protein or a polypeptide essentially corresponding thereto, derived from an edible fungus, comprising expression of such a gene, gene sequence or DNA fragment, for instance by culturing a cell containing this genetic material. In a further aspect, the invention relates to a regulatory region of a hydrophobin gene from an edible fungus, recombinant DNA material containing said region, as well as to a method for expressing genes in a fungus, preferably an edible fungus, in which said genes are brought to controlled expression under the control of said regulatory region. This method is preferably carried out using the genus <i>Agaricus</i>, especially the fruit bodies thereof.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TC	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

HYDROPHOBINS FROM EDIBLE FUNGI, GENES, NUCLEOTIDE SEQUENCES AND DNA-FRAGMENTS
ENCODING FOR SAID HYDROPHOBINS, AND EXPRESSION THEREOF

5

The present invention relates to hydrophobins of edible fungi and to genes, nucleotide sequences and DNA-fragments encoding for said hydrophobins.

10 Hydrophobins are a only recently discovered class of highly hydrophobic proteins and/or peptides occurring in certain fungi. So far, their existence has been shown in for instance the fungi *Schizophyllum commune*, *Aspergillus nidulans* and *Neurospora crassa*, vide the list of references mentioned hereinbelow.

15 In vivo, the hydrophobins from *Schizophyllum commune* are produced by the hyphae and/or fruit bodies. In emerging aerial hyphae, they accumulate at the hyphal surface to form SDS-insoluble complexes at the air/cell wall interface, which can only be destroyed by substances such as concentrated formic acid and trifluoroacetic acid. As such, the hydrophobins from *S. commune* make
20 the aerial hyphae highly water-resistant and water repellant.

The hydrophobins of *Aspergillus nidulans* and *Neurospora crassa* are produced by the conidiophores to form water resistant and water repellant coatings around the spores produced by the conidiophores.

25 Bell-Pedersen D. et al, Genes and Development 1992, pp. 2382-2394 describe the circadian clock-controlled gene, *ccg-2* from *Neurospora crassa*. This gene encodes for a fungal hydrophobin, said hydrophobin being a low-molecular-weight hydrophobic protein that provides for spore wall hydrophobicity and therefore ultimately for
30 spore dispersal.

The hydrophobins from the above mentioned fungi also show remarkable properties in vitro as for instance those of surfactants or emulgators. For example, the hydrophobins from *Schizophyllum commune* form self-assembling membranes around oil droplets with a
35 hydrophilic outer surface giving very stable emulsions. Hydrophobin Sc3p from *Schizophyllum commune* is reported to form an SDS-resistant complex at a water-gas interface that can for instance be used for

the formation of air vesicles.

A major drawback of the abovementioned hydrophobins, and other hydrophobins of other fungi so far described in the art, is however that they are derived from fungi that are considered non-edible and are sometimes even toxic to humans. Therefore, these hydrophobins cannot be regarded as safe for human consumption and/or be safely used in for instance personal care products, which could be an important field of applications for hydrophobins.

A first object of the invention is therefore to provide hydrophobins that can be considered safe for use in such products. Such hydrophobins could be derived from edible fungi, especially from the fruit bodies of edible mushrooms, such as *Agaricus bisporus*.

However, so far nothing is known about the presence, the structure or the properties of hydrophobins in edible mushrooms and their fruit bodies. The only fungi in which hydrophobins have so far been shown are the harmful and/or toxic fungi mentioned in the art.

Commercial use of hydrophobins from edible fungi would require the production thereof in sufficient quantities, e.g. by means of recombinant DNA-technology. This would require identifying, isolating, cloning and expressing the genes coding for these hydrophobins.

So far, only the genes coding for the different hydrophobins of harmful and/or toxic fungi such as *Schizophyllum commune* as well as *Aspergillus nidulans* and *Neurospora crassa* have been investigated.

J.G.H. Wessels, *Annu. Rev. Phytopathol.*, 1994, 32:413-37 reports that the *Sc3*, *Sc4* and *Sc1* genes from *Schizophyllum commune* show a homology of less than 40 %, despite the fact that these genes occur within the same organism. This reference also mentions that a comparison of the amino acid sequences encoded for by these genes with the hydrophobins *Rod A* from *Aspergillus nidulans* and *Eas* from *Neurospora crassa* shows an overall identity between these five hydrophobins of 11% and a similarity of 23%. Bell-Pedersen D. et al, loc.cit., mention an identity between the hydrophobins coded for by the *ccg-2*, *rodA* and *Sc3*-gene of 16%.

However, nothing is known about the genes coding for the

hydrophobins of edible mushrooms. In fact, the presence of such genes has so far not been shown in edible mushrooms such as *Agaricus bisporus*.

It is therefore another object of the invention to provide genes, nucleotide sequences or DNA-fragments coding for hydrophobins from edible fungi.

It is yet another object of the invention to provide recombinant DNA-material such as expression vectors and cloning vehicles containing these genes, nucleotide sequences and/or DNA-fragments.

Another object of the invention is the production of hydrophobins by expressing a gene, nucleotide sequence or DNA-fragment as described hereinabove. Further objects of the invention will be described hereinbelow.

The above and other objects are achieved in that the genes and the nucleotide sequences coding for the hydrophobins from edible fungi, especially from edible mushrooms from the genera *Agaricus*, are provided for the first time. Also, the amino acid sequences of the peptides/proteins for which these genes and nucleotide sequences code have been established, which for the first time provides said hydrophobins.

The invention therefore relates to a hydrophobin from an edible fungus and/or to a gene, nucleotide sequence or DNA-fragment coding for a ripening form of such a hydrophobin.

In particular, the invention relates to the hydrophobins from fungi of the genus *Agaricus*, more particular to the hydrophobins of *Agaricus bisporus*, the common mushroom, and especially to the hydrophobins from the fruit bodies of *Agaricus bisporus*, as well as to genes, nucleotide sequences or DNA-fragments coding for a ripening form of such a hydrophobin.

More in particular, the invention relates the nucleotide sequences and DNA-fragments derived from the hyp A, hyp B, hyp C and hyp D genes of *Agaricus bisporus*, the common mushroom, as well as to the amino acid sequences and the peptides/proteins - e.g. the hydrophobins - for which they code.

The hydrophobins of the invention will in general be a low molecular-weight cysteine rich protein, which usually a hydrophobic

amino terminus and an internal hydrophobic domain. They will in general contain 50-150, especially 90-120 amino acids, and 6-10, usually about 8 conserved cysteine-residues, as is characteristic for all hydrophobins described so far in the above mentioned references. However, the invention is not limited to a specific amino acid chain length or a specific number of cysteine residues.

It will however be understood that proteins and peptides with a similar structure and/or function as the hydrophobins will also be encompassed by the invention, as long as they are derived from an edible fungus and/or are not harmful to humans.

The term hydrophobins as used herein furthermore comprises all ripening forms of said hydrophobins, i.e. all the different forms in which the expressed proteins occur after expression of the genes, nucleotide sequences or DNA-fragments of the invention, such as the pre, prepro and pro forms thereof, as will be clear to a person skilled in the art.

As the hydrophobins of the invention are derived from edible mushrooms, they can be considered safe to humans or animals and can therefore be used both for human consumption, i.e. in food stuffs, as well as for products that come into contact with for instance the human skin, i.e. in personal care products.

Edible fungi and edible mushrooms are herein defined as fungi and mushrooms that can are not harmful and/or non-toxic to humans and/or animals and/or that can be eaten by humans and/or animals without deleterious effects. Preferably said fungi and mushrooms have the GRAS (Generally Regarded As Safe) status.

Such edible fungi and mushrooms are well known to a person skilled in the art, belonging to all classes of fungi, including the ascomycetes, basidiomycetes and the fungi imperfecti, from which the basidiomycetes are usually preferred because they generally form large edible fruit bodies. Preferred examples are the edible fungi from the group comprising the genera Agaricus, Pleurotus, Lentinus.

Preferably the hydrophobins of the invention are derived from the part of the fungus that is usually consumed by human beings as food, for instance the fruit body. It is also to be understood that if not all parts of a fungus are edible, the invention is only related to the hydrophobins derived from the fungus that are not

toxic and/harmful to humans or animals and that are preferably edible. Usually, these hydrophobins will be derived from the edible parts of the fungus, such as the fruit body, although the genes, nucleotide sequences or DNA-fragments coding for these hydrophobins may be derived from any part of the fungus.

The hydrophobins of the invention are preferably derived from fungi of the genus *Agaricus*, such as *A. arvensis*, *A. brunescens*, and *A. bitorquis*, and especially from *Agaricus bisporus*, the common mushroom.

The genes, nucleotide sequences and DNA-fragments coding for the hydrophobins of the invention are also derived from an edible fungus, more preferably from fungi of the genus *Agaricus*, and especially from *Agaricus bisporus*, the common mushroom. This assures that the hydrophobins for which they encode are indeed safe to use in foodstuffs and/or personal care products.

Four genes encoding for hydrophobins of *Agaricus bisporus* have been identified and have been named hyp A, hyp B, hyp C and hyp D respectively. Of these genes hyp A and hyp C are coded for on the same chromosome (chromosome III and IV, which are indistinguishable by CHEF-analysis), whereas hyp B and hyp D are coded for on chromosome XII and chromosome I respectively.

As is apparent from Figure 2, a DNA sequence showing a great deal of homology with the *Agaricus bisporus hypA* nucleotide sequence of figure 2 is present 2,5 kb downstream of *hypA*. Also, as shown in Figure 12, a DNA sequence showing a great deal of homology with the *Agaricus bisporus hypB* nucleotide sequence of figure 5 is present in the same organism. A part of the amino acid sequences of ripening forms of polypeptides having hydrophobic properties according to Figure 2 and 5 not only code for a part of HYPA and HYPB but also code for at least parts of other polypeptides having hydrophobic properties that can be derived from the same organism.

The invention in a preferred aspect therefore relates to a nucleotide sequence or DNA-fragment derived from the hyp A gene of *Agaricus bisporus*. In particular, this aspect of the invention relates to a nucleotide sequence or DNA-fragment containing the nucleotide sequence ID NO 1 shown in figure 2, as well as for the amino acid sequence for which it encodes, also shown in figure 2.

5 In another preferred aspect, the invention relates to a nucleotide sequence or DNA-fragment derived from the hyp B gene of *Agaricus bisporus*. In particular, this aspect of the invention relates to a nucleotide sequence ID NO 3 or DNA-fragment containing said nucleotide sequence as shown in figure 6, as well as for the amino acid sequence for which it encodes, also shown in figure 6.

10 In yet another preferred aspect, the invention relates to a nucleotide sequence or DNA-fragment derived from the hyp C gene of *Agaricus bisporus*. In particular, this aspect of the invention relates to a nucleotide sequence ID no 2 or DNA-fragment containing said nucleotide sequence as shown in figure 5, as well as for the amino acid sequence for which it encodes, also shown in figure 5.

15 Furthermore, the invention preferably relates to a nucleotide sequence or DNA-fragment derived from the hyp D gene of *Agaricus bisporus*, as well as for the amino acid sequence for which it encodes.

20 These preferred nucleotide sequences of the invention show a remarkably low homology of less than about 40 % with the hydrophobin encoding genes of *Schizophyllum commune* mentioned hereinabove. This means that probes for the *Schizophyllum*-hydrophobin genes cannot and could not be used for identifying or isolating the above *Agaricus* genes.

25 Also, the hydrophobins of the invention derived from the abovementioned nucleotide sequences show a remarkably low identity and similarity with the known hydrophobins from *Schizophyllum commune*, *Aspergillus nidulans* and *Neurospora crassa*. This is in correspondance with both the fact that they originate from different fungi (belonging to the ascomycetes and the basidiomycetes) as well as with their presence in different parts of the respective fungi (fruit body vs. aerial hyphae/spore coatings). Despite these differences, the hydrophobins of the invention also show the remarkable properties of said known hydrophobins, such as their hydrophobic properties, self-assembling properties, surface active properties and emulgating properties.

35 The preferred hydrophobins coded for by the above genes of *Agaricus bisporus* have been found to be very hydrophobic proteins that contain eight intramolecular sulfhydryl-bonds, that as such

provide for a major part of the tertiary structure of the protein. In *Agaricus bisporus*, these hydrophobins have now been shown to be part of the edible fruit body, where they form the water resistant and water repellant outer layer thereof. As such, they are now
5 considered major building blocks of the architecture of the fruit body, having an important role as a barrier-forming protein in its protection against water and against dehydration.

The hyp A and hyp B genes are for instance fruit body specific and show different expression levels during the various
10 stages of *Agaricus bisporus* fruit body development. The hyp B gene shows highest expression during the early stages of fruit body development whereas the hyp A gene shows high mRNA expression levels from the onset of fruiting at least until the growth stage in which the fruit bodies are used for consumption.

15 The invention is however not restricted to a certain mechanism of action or structural purpose of the hydrophobins of the invention within the fungus or fruit body.

It is to be understood that the invention not only provides for the hydrophobins as they naturally occur in edible fungi, but
20 also for proteins and peptides that "essentially correspond" to the hydrophobins of the invention, i.e. that contain a large part of the protein structure or amino acid sequence of said hydrophobins.

These can be:

- proteins having the same amino acid sequence as the
25 hydrophobins of the invention, however with a part of the peptide chain missing, preferably the N-terminal or -COOH-terminal part thereof.
- proteins having the same amino acid sequence as the
30 hydrophobins of the invention, in which one or more amino acids have been deleted and/or replaced by other amino acids.
- proteins having the same amino acid sequence as the
hydrophobins of the invention, in which part of the amino acid sequence has been inversed, i.e. is present in reversed order compared to the naturally occurring hydrophobin.
- 35 - proteins having the same amino acid sequence as the hydrophobins of the invention, to which one or more amino acids have been added,

- or a combination thereof,
as will be clear to a person skilled in the art.

5 In general, a protein will be considered to essentially correspond to a hydrophobin of the invention when it contains at least 50%, preferably at least 70% and more preferably at least 90% of the amino acid sequence of the hydrophobin of the invention, as a closed sequence or as separate fragments within the total amino acid sequence.

10 These proteins or polypeptides preferably have essentially the same tertiary and/or quaternary structure, as well as the same properties as the naturally occurring hydrophobins. More preferably, they contain the same number of sulfhydryl bonds as the naturally occurring hydrophobins.

15 It is to be understood however that these proteins or polypeptides may also be specifically engineered or tailored for obtaining desired and/or improved properties, such as improved hydrophobicity, improved stability and/or improved surfactant/emulgator properties, i.e. by adding further cysteine residues, adding/replacing or deleting amino acids with hydrophilic or hydrophobic residues etc.

20 The above proteins and peptides can be prepared by a person skilled in the art in a manner known per se, for instance by deleting or adding one or more amino acids from/to the hydrophobins of the invention.

25 However, in general they will be produced by recombinant DNA-technology, i.e. by expressing a nucleotide sequence or DNA-fragment coding for such a peptide or protein, and these nucleotide sequences and DNA-fragments are also encompassed by the nucleotide sequences and DNA-fragments of the invention. Usually, these nucleotide sequences and DNA-fragments will be "genetic variations" of the genes, nucleotide sequences and DNA-fragments of the invention, as further defined hereinbelow.

30 In a further preferred embodiment, the invention therefore also relates to peptides and proteins that essentially correspond to the hydrophobins coded for by the hyp A, hyp B, hyp C and hyp D genes of *Agaricus bisporus*, i.e. with an amino acid sequence that essentially corresponds to one of the amino acid sequences shown in

figures 1-3.

All these peptides and proteins that essentially correspond to the hydrophobins of the invention, in all their ripening forms as defined herein, are to be understood as being comprised by the term hydrophobin as used hereinbelow.

Furthermore, it is to be understood that the invention also comprises homologues of the above genes, nucleotide sequences or DNA-fragments, i.e. sequences that code for the same amino acid sequence or peptide/protein but with a different nucleotide sequence due to the degeneracy of the genetic code, as will be clear to a person skilled in the art. In particular, the invention expressly includes homologues of the nucleotide sequences shown in figures 1-3. Also, the invention comprises genetic variants of these genes, nucleotide sequences or DNA-fragments, as mentioned hereinabove and/or as defined hereinbelow.

The hydrophobins of the invention may advantageously be used in industrial processes, for example as emulgators, thickeners or surfactants, for example for giving hydrophilic properties to hydrophobic surfaces or for improving water-resistance of hydrophilic substrates.

In the field of food technology, they may also be used as surfactants or emulgators, or they may be used with advantage for improving the storage properties of various foodstuffs that are water sensitive, such as biscuits, candies and chocolate, or for lowering the water activity of foodstuffs that become easily infected by micro-organisms.

In the field of personal care products, the hydrophobins of the invention can be used as emulgators for the preparation of both oil-in-water as well as water-in-oil emulsions, as well as higher emulsions, such as W/O/W and/or O/W/O emulsions, wherein the excellent stability of the hydrophobin complexes formed on the oil/water interfaces, especially against other surfactants such as SDS, may be of particular value. Such emulsions may be part of pharmaceutical preparations, especially preparations for topical application such as ointments and creams. Also, they may be used in for instance shampoos and conditioners in which their property of forming a water repellant layer can be used for protecting the human

skin or dehydration.

The invention therefore further relates to foodstuffs and compositions, especially pharmaceutical preparations and/or personal care preparations, comprising a hydrophobin of the invention, or a peptide or protein essentially corresponding thereto.

The hydrophobins of the invention may further advantageously be applied in the field of environmental technology, for instance in the cleaning of contaminated soil or water, especially in the soil or water contaminated with oil-like substances. In these applications, the biological compatability of the hydrophobins of the invention is a further advantage.

The present invention in a further aspect also relates to recombinant DNA material comprising

- a) at least part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus,
- b) a genetic variant of a nucleotide sequence according to a); or
- c) a nucleotide sequence capable of hybridizing to either of the nucleotide sequences a) or b).

The recombinant DNA material comprising a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus can comprise a nucleotide sequence derivable from an organism that is homologous to the expression host cell into which cell said nucleotide sequence is incorporated or said nucleotide sequence can be heterologous to the expression host cell.

The expression of the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus can be regulated by operably linking said nucleotide sequence to regulatory sequences that control a gene native to the edible fungus from which said nucleotide sequence has been derived. The regulatory sequences can also be foreign i.e. derived from an organism belonging to a different strain, variety, genus or group of organisms than the edible fungus from which the nucleotide sequence encoding a hydrophobin of an edible fungus has been derived. The regulatory regions can be regulatory regions of a hydrophobin gene or regulatory regions of other genes.

Another preferred embodiment of the invention is related to a cell capable of overexpression and secretion of a ripening form of a

hydrophobin of an edible fungus, preferably a mature form.

It is yet a further aspect of the present invention to provide a method for the production of a ripening form of a hydrophobin of an edible fungus, which may be used in an industrial process, in the field of environmental technology, in the field of food technology and/or in the field of personal products as described hereinabove, or in any other field envisaged.

The invention will now be further illustrated by means of the description hereinbelow as well as by the figures, in which:

- 10 Fig. 1: Restriction map of the genomic DNA of *A. bisporus* in the region comprising the *hypA* and *hypC* genes.
- Fig. 2: Nucleotide sequence of cDNA derived from mRNA expressing the *A.bisporus hypA* gene and amino acid sequences derived therefrom. (SEQ ID NO: 1)
- 15 Fig. 3: Nucleotide sequence of genomic DNA of the *A.bisporus hypA* and *hypC* genes. (SEQ ID NO: 2)
- Fig. 4: Restriction map of the genomic DNA of *A. bisporus* in the region comprising the *hypB* gene.
- Fig. 5: Nucleotide sequence of cDNA derived from mRNA expressing the *A.bisporus hypB* gene and amino acid sequences derived therefrom. (SEQ ID NO: 3)
- 20 Fig. 6: Nucleotide sequence of genomic DNA of the *A.bisporus hypB* gene. (SEQ ID NO: 4)
- Fig. 7: Partial restriction enzyme map of the hybridizing phage clone 10 comprising the *A.bisporus hypD* gene.
- 25 Fig. 8: Map of plasmid pIM3100 comprising cDNA encoding the *A.bisporus hypA* gene.
- Fig. 9: Map of plasmid pIM3101 comprising cDNA encoding the *A.bisporus hypB* gene.
- 30 Fig. 10: Map of plasmid pIM3104 comprising the *A.bisporus hypA* and *hypC* genes and regulatory sequences.
- Fig. 11: Map of plasmid pIM3106 comprising the *A.bisporus hypB* gene and regulatory sequences.
- Fig. 12: CHEF-analysis of *hypB* and *hypD* genes.
- 35 lane 1: *A. bisporus* strain 39
lane 2: *A. bisporus* strain 97
A: probed with a 1 kb *Bam*HI- *Eco*Ri fragment of phage

1 located 2 kb upstream of *hypB*.

B: probed with the insert of pIM3101.

C: probed with a 1.2 kb *EcoRI-BamHI* fragment of phage 10.

5 With regard to the SEQ ID's it is remarked that these contain both the cDNA (starting with the methionine-encoding/ATG start codon and ending with a stop codon - TAG, TAA, TGA - known per se) well as the regulatory region of the specific gene, containing the promoter controlling said gene, as will be appreciated by a man skilled in the art.

10 In one aspect, the present invention is directed at a recombinant DNA material comprising at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus and genetic variants thereof. Usually, and preferably, said
15 recombinant DNA-material will comprise at least a part of a gene, nucleotide sequence or DNA-fragment as described hereinabove, or a genetic variant or a homolog due to the degeneracy of the genetic code thereof.

20 The term "recombinant DNA material" can comprise a DNA molecule, or a mixture of various DNA fragments/ molecules. It also includes for instance expression vectors and cloning vehicles, such as plasmids.

25 The term "genetic variants" as used herein includes hybrid DNA sequences comprising at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus optionally coupled to regulatory regions such as promoter, secretion and terminator signals originating from homologous or heterologous organisms.

30 The term "genetic variants" also includes DNA sequences encoding mutant hydrophobins and degenerate DNA sequences encoding polypeptides that essentially correspond to the hydrophobins of the invention as defined hereinabove. These genetic variants may be obtained in a manner known to a man skilled in the art, for instance by adding or deleting one or more codons coding for one or more
35 amino acids, either at the beginning or the end of the of the nucleotide sequence or somewhere inbetween, or by replacing one or more codons coding for an amino acid with one or more other codons

coding for one or more other amino acids, for instance by means of point mutation.

Preferably, these genetic variants show a homology of more than 40%, preferably more than 80%, with the nucleotide sequence derived from the edible fungus.

The present invention also includes recombinant DNA material comprising at least a part of a nucleotide sequence capable of hybridizing under low stringency conditions to at least a part of the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus and genetic variants thereof as described above which may differ in codon sequence due to the degeneracy of the genetic code or cross species variation.

The term "ripening form" refers to any of the different forms in which an enzyme may occur after expression of the associated gene. More in particular it refers to both the naturally and not naturally occurring mature form of an enzyme that can result after cleavage of a "leader" peptide and also to any form of an enzyme still comprising a "leader" peptide in any form. In general a "leader peptide" can be a prepro peptide, a pre peptide or a pro peptide.

The recombinant DNA material according to the invention can comprise at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus wherein said nucleotide sequence can be derived from any edible fungus, such as the fungi mentioned hereinabove.

A more concrete preferred embodiment of this aspect of the invention is recombinant DNA material comprising at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus with an amino acid sequence as shown in SEQ ID's 1, 2 or 3 and even more concretely a recombinant DNA material comprising at least a part of the nucleotide sequence.

A more concrete preferred embodiment of this aspect of the invention is recombinant DNA material comprising at least a part of a nucleotide sequence encoding a ripening form of a polypeptide having hydrophobic properties with an amino acid sequence as shown in Figures 2 or 5 and even more concretely a recombinant DNA material comprising at least a part of the nucleotide sequence

encoding a polypeptide having hydrophobic properties as shown in Figure 3 or 6. The genetic variants of the nucleotide sequence of Figure 3 or 6, including sequences encoding mutant polypeptides with hydrophobic properties and degenerate nucleotide sequences coding for polypeptides wherein the hydrophobic properties are retained are also part of the invention, as are nucleotide sequences capable of hybridizing to at least a part of the nucleotide sequences encoding a polypeptide having hydrophobic properties as shown in Figure 3 and 6 and genetic variants thereof (as described above), wherein said nucleotide sequences may differ in codon sequence due to the degeneracy of the genetic code or cross species variation. The cDNA sequences of Figure 2 and 5, encoding polypeptides having hydrophobic properties are obtained by screening an *Agaricus bisporus* mixed primordia - fruit body cDNA library for highly expressed genes.

The genetic variants of the nucleotide sequences of SEQ ID's, as derived from *Agaricus bisporus*, are also part of the invention, as are nucleotide sequences capable of hybridizing to at least a part of the nucleotide sequences encoding a hydrophobin of an edible fungus as shown in SEQ ID's 1, 2 and 3 and genetic variants thereof (as described above), wherein said nucleotide sequences may differ in codon sequence due to the degeneracy of the genetic code or cross species variations.

Sequences showing a great deal of homology with the nucleotide sequences of SEQ ID's 1, 2 or 3 derived from *Agaricus bisporus* may be present in other edible fungi, especially in other species from the genus *Agaricus*, such as the ones mentioned hereinabove. Such a nucleotide sequence from another fungus can be selected due to the fact that at least a part of the nucleotide sequences of SEQ ID's 1, 2 or 3 as derived from *Agaricus bisporus* or a corresponding degenerate DNA sequence derived from the amino acid sequence of SEQ ID's 1 2 or 3 or derived from an equivalent amino acid sequence can hybridize with genetic material of said other fungus. The hybridizing part of the genetic material of the other organism comprises at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus.

Using the process for recovering such a nucleotide sequence

as given in the examples, a person skilled in the art can derive a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus from another organism.

5 These and other genes, nucleotide sequences and/or DNA-fragments, especially those coding for hydrophobins that are formed during the emergence of the fruit bodies and/or that form part of the -preferably edible- fruit bodies can also be obtained by the procedure put forward in the Examples with respect to *Agaricus bisporus*, said procedure being based on the abundance of mRNA and
10 transcription thereof during said phase of the life cycle of the fungus.

The recombinant DNA material according to the invention can be used to express a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus or the recombinant DNA material
15 can be used as a probe or a primer for detection or production of genetic material encoding at least a part of a ripening form of a hydrophobin in a fungus, preferably an edible fungus.

The recombinant DNA material according to the invention can comprise regulatory regions, i.e. regulatory regions native to the
20 fungus from which the hydrophobin is derived or regulatory regions foreign to the fungus from which the nucleotide sequence encoding the hydrophobin of an edible fungus is derived operably linked to said nucleotide sequence, for instance bacterial, yeast, plant or animal regulatory regions. In this instance the regulatory regions
25 will usually be regulatory regions that regulate genes coding for other peptides than hydrophobins, as bacteria, yeasts, plants and animals are not known to contain and/or produce hydrophobins.

It is also possible that the recombinant DNA material according to the invention comprises regulatory regions from other
30 fungi than the fungus from which the hydrophobin gene has been derived, said regulatory regions themselves regulating hydrophobin genes and/or regulating other genes in their respective native fungus.

The selection of a desirable regulatory region will be
35 obvious to one skilled in the art and will for example depend on the host cell into which the recombinant DNA material according to the invention is introduced. If a heterologous expression host is

preferred, meaning that the nucleotide sequence encoding a hydrophobin of an edible fungus is derived from another strain of organism than the host cell (e.g. a different strain, variety, species, genus, family, order, class, division or kingdom) the regulatory region is preferably a regulatory region derived from an organism similar to or equal to the expression host. For example, if the expression host is a yeast cell, then the regulatory region will be derived from a yeast cell. The regulatory region need not however necessarily be derived from the same strain or the same genus as the host cell, i.e. a yeast cell. The selection of a yeast cell promoter in this instance is required to enable expression of the nucleotide sequence.

A regulatory region operably linked to a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus in the recombinant DNA material according to the invention can be e.g. a constitutive promoter or an inducible promoter. Especially suited are constitutive promoters derived from genes encoding enzymes involved in the glycolytic pathway.

An example of a recombinant DNA material according to the invention comprising a strong constitutive promoter operably linked to the nucleotide sequence encoding a ripening form of a hydrophobin from an edible fungus is a recombinant DNA material wherein said promoter is the glyceraldehyde-3-phosphate dehydrogenase (gpdA) promoter. This promoter is preferred for constitutive expression when recombinant DNA material according to the invention is expressed in a fungal expression host. Other examples are the phosphoglycerate kinase (pgk) promoter, the pyruvate kinase (pki) promoter, TPI, the triose phosphate isomerase (tpi) promoter, the APC synthetase subunit g (oliC) promoter and the acetamidase (amdS) promoter.

Examples of recombinant DNA material according to the invention comprising inducible promoters operably linked to the nucleotide sequence encoding a ripening form of a hydrophobin from an edible fungus activity are recombinant DNA materials, wherein said inducible promoters are selected from the promoters of the following genes: xylanase A (xylA), glucoamylase A (glaA), cellobiohydrolase (cbh), amylase (amy), invertase (suc) and alcohol

dehydrogenase *alcA*, TAKA amylase and amyloglucosidase (AGT). Preferably the inducible xylanase A promoter is selected.

Examples of recombinant DNA material according to the invention comprising strong yeast promoters operably linked to the nucleotide sequence encoding a ripening form of hydrophobin from an edible fungus activity are recombinant DNA materials, wherein said yeast promoters are selected from the promoters of the following genes: alcohol dehydrogenase, lactase, 3-phosphoglycerate kinase, triose phosphate isomerase, α -D-galactose-phosphate uridyl transferase (Gal7) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Examples of recombinant DNA material according to the invention comprising bacterial promoters operably linked to the nucleotide sequence encoding a ripening form of hydrophobin from an edible fungus activity are recombinant DNA materials, wherein said bacterial promoters are selected from the promoters of the following genes: α -amylase, SP02 and extracellular proteases.

If a heterologous expression host is a yeast or a bacterial strain a recombinant DNA material according to the invention comprising an uninterrupted (intronless) nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus is preferred. This preference stems from the fact that the possibility that the heterologous host does not recognize splicing signals residing on the recombinant DNA material can thus be avoided. Such an uninterrupted nucleotide sequence may be obtained from a cDNA library constructed from RNA isolated from cells expressing a nucleotide sequence encoding a ripening form of a polypeptide with hydrophobin from an edible fungus. Alternatively an uninterrupted nucleotide sequence may be obtained by applying one or more polymerase chain reactions using suitable primers, so as to precisely remove the introns, using genomic DNA as a template, as is known to a person skilled in the art.

For the expression in yeast such as *Saccharomyces cerevisiae* it is preferable that the introns are removed and that the fungal leader sequence is replaced by a signal sequence suitable for yeast such as the signal sequence of the invertase gene ensuring correct processing and secretion of the mature polypeptide.

The removal of introns is necessary for expression in bacteria such as Bacillus subtilis. In this case for example the α -amylase signal sequence can be used as signal sequence.

5 A preferred embodiment of recombinant DNA material according to the invention comprises a selection marker. Such a selection marker serves to discriminate host cells into which the recombinant DNA material has been introduced from cells that do not comprise said recombinant DNA material. This selection marker provided with the appropriate regulatory sequences may reside on the same DNA
10 fragment containing the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus or can be present on a separate fragment. In the latter case a co-transformation must be performed with the various components of the recombinant DNA material according to the invention. The ratio of expression component
15 (containing the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus) / selection component (with the selection marker) can be adjusted in such a manner that a high percentage of the selected cells comprising the selection component have also incorporated the expression component. The term re-
20 combinant DNA material as used herein therefore comprises one or more recombinant DNA fragments, wherein the selection marker can be incorporated on the same recombinant DNA molecule as the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus or on a different recombinant DNA fragment.

25 Very often filamentous fungi are transformed through co-transformation. For example a pyrA^- strain (pyrA = orotidine-5'-phosphate decarboxylase) can be used as host cell and the recombinant DNA material according to the invention will comprise a DNA molecule comprising the nucleotide sequence encoding a ripening
30 form of a hydrophobin of an edible fungus and another DNA molecule comprising the pyrA gene. After transformation of the pyrA^- strain any resulting pyrA^+ strain will obviously have incorporated some recombinant DNA material and will most probably also comprise the nucleotide sequence encoding a ripening form of a hydrophobin of an
35 edible fungus. Very often such co-transformation will lead to incorporation of the component of recombinant DNA material according to the invention comprising the nucleotide sequence encoding a

ripening form of a hydrophobin of an edible fungus per host cell in multiple copies (multicopy incorporation). This is a well-known route for producing multicopy transformants in general.

5 Other well-known selection systems for industrial microorganisms are those formed by the group of selection markers which do not require a mutation in the host organism. Examples of fungal selection markers are the genes for acetamidase (*amdS*), ATP-synthetase, subunit 9 (*oliC*) and benomyl resistance (*benA*). Another example of a fungal selection marker is the nitrate reductase system. Exemplary of non-fungal selection markers are the *g418* resistance gene (yeast), the ampicillin resistance gene (*E. coli*) and the neomycin resistance gene (*Bacillus*), a gene conferring resistance to hygromycin (*hph*) or a gene conferring resistance to fleomycin (*Ble*).

15 Suitable transformation methods and suitable expression vectors provided with e.g. a suitable transcription promoter, suitable transcription termination signals and suitable marker genes for selecting transformed cells are already known for many organisms including different bacterial, yeast, fungal and plant species. Reference may be made for yeast for example to Tagima et al. Yeast 1, 67-77, 1985, which shows expression of a foreign gene under control of the *gal7* promoter inducible by galactose in yeast and for *Bacillus subtilis* for example in EP-A-0,157,441 describing a plasmid pNS48 containing the SPO2 promoter as an expression vector. For the possibilities in these and other organisms reference is made to the general literature.

20 Overexpression of a ripening form of a hydrophobin of an edible fungus may be achieved by the incorporation of recombinant DNA material according to the invention in an expression host, said recombinant DNA material comprising one or more regulatory regions (selected for example from promoter and terminator regions) which serve to increase expression levels of the polypeptide of interest from said expression host. If desired the polypeptide of interest can be secreted from the expression host. This can be achieved by incorporating recombinant DNA material according to the invention as described further comprising at least one signal sequence (e.g. a pre or prepro sequence).

The present invention is not only directed at the recombinant DNA material comprising at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus in the various embodiments as described above but is also directed at a cell comprising at least a part of said recombinant DNA material, said cell being capable of expression of said nucleotide sequence.

Progeny of an expression host comprising recombinant DNA material according to the invention is also embraced by the present invention.

Preferably a cell according to the invention will be capable of overexpression of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus. Within the context of the present invention overexpression is defined as the expression of the ripening form of a hydrophobin of an edible fungus at levels above those ordinarily encountered under the same conditions in the native organism from which said polypeptide originates. In the same context overexpression also covers the expression of the ripening form of a hydrophobin of an edible fungus in an organism other than the organism from which the nucleotide sequence comprised on the recombinant DNA material according to the invention can be derived, a so called heterologous organism. The heterologous host organism does not normally produce such a ripening form of a hydrophobin of an edible fungus at appreciable levels and the heterologous organism is therefore only capable of such production after introduction of the recombinant DNA material according to the invention.

As already stated, overexpression of a ripening form of a hydrophobin of an edible fungus may be achieved by incorporation of recombinant DNA material according to the invention.

In order to obtain overexpression recombinant DNA material according to the invention can be incorporated in a homologous expression host. The term "homologous expression host" means that the non transformed expression host belongs to the same strain or species as the organism from which the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus that is comprised on the recombinant DNA material according to the invention has been derived.

Introduction of the recombinant DNA material according to the

invention into a homologous expression host will result in the expression host comprising at least two nucleotide sequences encoding a ripening form of hydrophobin of an edible fungus, becoming a so-called multicopy transformant.

5 The overexpression can be further achieved by the introduction of the recombinant DNA material according to the invention into a host belonging to a strain other than the strain from which the nucleotide sequence encoding a ripening form of hydrophobin of an edible fungus was isolated a so-called
10 heterologous host, such that the resulting expression host comprises a nucleotide sequence encoding a ripening form of hydrophobin of an edible fungus in increased gene copy numbers, becoming a so-called multicopy transformant.

 The methods generally known for obtaining multicopy
15 transformants can be used. The recombinant DNA material according to the invention therefore comprises any embodiment required for obtaining a multicopy transformant comprising multiple copies of the nucleotide sequence encoding a ripening form of hydrophobin of an edible fungus.

20 The overexpression can also be achieved by the introduction of the recombinant DNA material according to the invention in the various embodiments already described into a host cell such that the host cell comprises the nucleotide sequence encoding a ripening form of hydrophobin of an edible fungus under the control of a regulatory
25 region other than the native regulatory region for the hydrophobin from an edible fungus gene in the organism from which said nucleotide sequence is derived, said other regulatory region preferably being more efficient than the native regulatory region. The invention is also directed at recombinant DNA material in any of
30 the various embodiments described further comprising a regulatory region other than the native regulatory region for the hydrophobin from an edible fungus gene in the organism from which said nucleotide sequence is derived. Such a host cell can be either homologous or heterologous. The host cell can comprise one or more
35 copies of the nucleotide sequence encoding a ripening form of hydrophobin of an edible fungus comprised on the recombinant DNA material according to the invention.

In some instances it can be preferable to introduce recombinant DNA material according to the invention in such a manner that said recombinant DNA material is integrated in the chromosomal DNA of the host cell. In fungal cells chromosomal integration always takes place in successful transformations. No plasmid DNA is maintained. In yeast both plasmids and integrated DNA can be maintained satisfactorily.

It is possible to introduce recombinant DNA material into the host cell such that the genetic properties that are introduced are located on extra-chromosomal DNA most often called "plasmids". Plasmids have the advantage that they exist normally in the cell in multiple copies which also means that a certain gene located on such a plasmid exists in the cell in multicopy form which may result in a higher expression of the proteins encoded by the genes. However, the disadvantage of plasmids is that they can be unstable resulting in a possible loss of the plasmids from the cells at a certain stage. The loss of a plasmid can be prevented by using a plasmid comprising at least one stretch of nucleotides capable of hybridizing with chromosomal DNA of the non-transformed host cell enabling said vector to integrate stably into the chromosome of said host cell after transformation. Use of a stretch of homologous DNA that is already present in multiple copies in the chromosomal DNA will lead to multicopy insertion of the vector DNA resulting in integrated multimeric DNA comprising one or more copies of the nucleotide sequence encoding a ripening form of a hydrophobin from an edible fungus. Another prerequisite for a vector resulting in integrated DNA in the chromosomal DNA is that the vector does not comprise a functional replicon as the vector must be unable to maintain itself in the host cell unless it is integrated.

The stretch of nucleotides enabling integration is preferably derivable from DNA that comprises at least part of a non-essential portion of the chromosome of a non-transformed host cell (in this instance the term "derivable from" implies that the stretch of nucleotides in the vector according to the invention must show enough homology with the chromosomal DNA to enable hybridization for an integration event to occur). The integration of the vector will subsequently take place in said non-essential portion of the

chromosome of the host cell and will not lead to the loss of essential function of the host cell. It is preferable for the integration to take place in a non-essential selectable gene of the chromosome of the non-transformed host cell. This can be subsequently a selection criterium for transformed host cells.

In the case of fungal cells it is only possible to successfully obtain transformants having DNA integrated in the chromosomal fungal DNA as plasmids cannot be maintained in such cells. In fungal cells it is not even necessary to include homologous chromosomal DNA as multicopy integration takes place without said homologous DNA. In the case of yeast cells it is optional to have the desired DNA in the transformant either as a plasmid or as integrated DNA. For integration in yeast cells DNA sequences homologous to chromosomal DNA must be present.

A preferred embodiment of the invention is directed at a cell comprising recombinant DNA material according to the invention in any of the embodiments described, wherein said cell is capable of secreting a ripening form in particular capable of secreting a mature form of a polypeptide with hydrophobin from an edible fungus as encoded by said recombinant DNA material. It is often desirable for the ripening form of a hydrophobin of an edible fungus to be secreted from the expression host into the culture medium as said polypeptide may be more easily recovered from the medium than from the cell. Preferably the mature form of the hydrophobin from an edible fungus will be secreted into the culture medium.

The term "secretion" in the subject invention comprises the polypeptide crossing a cell wall or a cell membrane. The polypeptide can pass such a cell wall or membrane into the culture medium but can also remain attached to said cell wall or cell membrane. The polypeptide can also pass a cell membrane into the periplasmic space and not into the culture medium. The processing c.q. secretion route to be followed by the ripening form of a hydrophobin of an edible fungus will depend on the selected host cell and the composition of the recombinant DNA material according to the invention. Most preferably, however, the polypeptide will be secreted into the culture medium. However, due to the extraordinary properties of the hydrophobins secretion thereof may not always be possible, and

intracellular excretion of excretion at or near the cell wall or membrane, or on the outside thereof, followed by destruction of the cell, for example by lysis or sonication, and isolation of the hydrophobins in a manner known per se is also included by the invention. With regard to the lysis of the cells and/or the isolation of the hydrophobins, the excellent stability of the hydrophobin complexes, especially against SDS and other surfactants, may be used with advantage.

The cell according to the invention can comprise recombinant DNA material in any of the various embodiments described further comprising DNA encoding the native leader sequence (pre or prepro) of the hydrophobin of an edible fungus. In another embodiment the cell according to the invention can comprise recombinant DNA material further comprising DNA encoding for foreign leader sequences (pre or prepro) instead of the native leader sequences. The invention is also directed at recombinant DNA material comprising DNA encoding the mature hydrophobin of an edible fungus coupled to DNA encoding a leader sequence foreign to the hydrophobin of an edible fungus.

An increase in the expression of a hydrophobin of an edible fungus can result in the production of polypeptide levels beyond those the expression host is capable of processing and secreting resulting in a build up of polypeptide product within the host cell creating a bottle neck in the transport of the polypeptide through the cell membrane or cell wall. Accordingly the present invention is also directed at a cell comprising recombinant DNA material in any of the various embodiments described comprising heterologous signal sequences to provide for the most efficient secretion of the hydrophobin from an edible fungus from the chosen expression host and the invention is also directed at said recombinant DNA material.

A heterologous secretion signal sequence may be chosen such that it is derived from the same strain as the organism from which the other regulatory regions of the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus have been derived, preferably from the same gene. For example the signal of the highly secreted amyloglucosidase protein may be used in combination with the amyloglucosidase promoter itself as well as in

combination with other promoters.

Examples of preferred heterologous secretion signal sequences are those originating from the glucoamylase A or xylanase A gene for fungi, the invertase gene for yeast and the α -amylase gene for Bacillus.

Hybrid secretion sequences may also advantageously be used within the context of the present invention.

In general terminators of transcription are not considered to be critical elements for the overexpression of genes. If desired, a terminator of transcription may be selected from the same gene as the promoter or alternatively the homologous terminator may be employed. In fact any terminator can be employed.

Factors such as size (molecular weight) the possible need for glycosylation or the desirability of the secretion over the cell membrane or cell wall or into the medium of the hydrophobin from an edible fungus play an important role in the selection of the expression host.

Partly depending on the selected host cell the nucleotide sequence encoding a hydrophobin of an edible fungus will be used either with or without introns occurring in said DNA sequence either with its own promoter and/or transcription termination signals or originating from another gene and either with its own leader sequence or with a signal sequence originating from another gene.

In principle the invention knows no special limitations with respect to the nature of the cells comprising recombinant DNA material according to the invention. Cells according to the invention may be important as agents for multiplying the recombinant DNA material or as agents for producing a ripening form of a hydrophobin of an edible fungus.

Those expression hosts capable of overexpression of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus are preferred. In particular an expression host cell capable of secretion of a ripening form of hydrophobin of an edible fungus is preferred.

The expression hosts are preferably selected from the group consisting of bacterial cells, fungal cells, yeast cells and plant cells.

Preferred examples of eminently suited host cells are

- 5 a) fungal cells, in particular filamentous fungal cells, such as a fungal cell from the group comprising the genera Aspergillus, Trichoderma, Neurospora, Penicillium and Mucor.
Examples of particular species that are suitable as host cell are fungal cells of one of the species Aspergillus niger, Aspergillus awamori, Aspergillus oryzae, Aspergillus sojae, Aspergillus tubigensis, Aspergillus aculeatus, Aspergillus japonicus, Trichoderma reesei and Trichoderma viride;
- 10 b) yeast cells, for example of the genera Saccharomyces, Kluyveromyces, Hansenula and Pichia, in particular yeast cells of one of the species Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Kluyveromyces lactis, Kluyveromyces marxianus, Hansenula polymorpha and Pichia pastoris;
- 15 c) plant cells of a plant genus selected for example from the group consisting of wheat, barley, oats, maize, pea, potato and tobacco such as plant cells of one of the species Solanum tuberosum and Nicotiana tabacum; and
- 20 d) bacterial cells, preferably gram positive bacterial cells, for example of one of the bacterial genera Bacillus, Lactobacillus and Streptococcus such as bacteria of the species Bacillus subtilis or Bacillus licheniformis.

25 The host cell to be selected for recombinant DNA material according to the invention will amongst others depend on the application for which the resulting hydrophobin of an edible fungus is destined.

30 A preferred cell according to the invention is a foodgrade cell. This preference stems from the fact that products of such foodgrade cells can be used in processes for producing foodstuffs. Bacteria from the genus Bacillus are very suitable as expression host cells because of their capability to secrete proteins into the culture medium. Alternatively a host selected from the group of yeasts or fungi may be preferred. In some instances yeast cells are
35 easier to manipulate than fungal cells. However, some proteins are either poorly secreted from the yeast cell or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these and

other instances a fungal host organism can be selected. A fungal host is often suitable if it has GRAS status (GRAS = generally regarded as safe). In general, eukaryotic hosts have been found to have a high productivity of secreted active polypeptides. In fact fungal hosts are very often used in industrial processes, particularly suitable examples of a host cell are therefore Aspergillus niger and Aspergillus niger var. awamori. These particular species of Aspergillus have previously been demonstrated to be excellent host cells for industrially producing enzymes. A person skilled in the art is able to obtain multicopy transformants of these species.

In the case of polypeptide production it is possible to use the expression host cell to produce polypeptide and to subsequently either isolate the polypeptide from the culture medium or use the medium containing the polypeptide as such after removal of the cells. It is even possible to use the cells themselves to produce the polypeptide in situ in the process for which the hydrophobin of an edible fungus is required. In the preparation of foodstuffs such a host strain that is to be used directly can only be used if it is a food grade host strain. In connection with bread making for example yeast strains that have been genetically manipulated in accordance with the present invention can be used directly.

If the polypeptide is required in extremely purified forms or if particular contaminants are deleterious to the application of the resulting polypeptide, the expression host cell can be selected to avoid such problems.

The subject invention is also directed at a ripening form of a polypeptide with hydrophobin from an edible fungus activity wherein said ripening form is obtainable by expression of the recombinant DNA material according to the invention. The invention is preferably directed at a mature form of a polypeptide with hydrophobin from an edible fungus activity as no further treatment of said polypeptide is necessary before using said polypeptide in a desired process. In particular the invention is directed at a ripening form of a polypeptide as encoded by a part of the amino acid sequence of Figure 1, 2 or 3. A ripening form of a hydrophobin of an edible fungus, said ripening form being encoded by a part of

any equivalent amino acid sequence encoding a polypeptide with an equivalent tertiary structure having hydrophobin from an edible fungus also forms part of the invention.

5 The invention is also directed at a process for producing a ripening form of a hydrophobin of an edible fungus comprising the culture of a cell as previously described in the specification and optionally isolation of the resulting ripening form of a hydrophobin of an edible fungus. The expression of the polypeptide with hydrophobin from an edible fungus activity can be effected by
10 culturing expression host cells that have been transformed with the recombinant DNA material comprising a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus in a conventional nutrient fermentation medium.

15 The fermentation medium can comprise an ordinary culture medium containing a carbon source, a nitrogen source, an organic nitrogen source and inorganic nutrient sources. The selection of the appropriate medium may be based on the choice of expression host and/or based on the regulatory requirements of the recombinant DNA material. Such media are well-known to those skilled in the art. The
20 medium may, if desired, contain additional components favouring the transformed expression host over other potentially contaminating microorganisms. In the case of production of the hydrophobin of an edible fungus for food processing such additional components are necessarily also food grade.

25 After fermentation the cells can be removed from the fermentation broth by means of centrifugation or filtration. Depending on whether the host cell has secreted the hydrophobin of an edible fungus into the medium or whether said polypeptide is still connected to the host cell in some way either in the
30 cytoplasm, in the periplasmic space or attached to or in the membrane or cell wall, the cells can undergo further treatment to obtain the polypeptide.

In the latter case, where the polypeptide is still connected to the cell in some manner, recovery of the polypeptide can for
35 example be accomplished as described in US 4,894,340 or US 4,632,905 by rupturing the cells for example by high pressure disruption, sonication, enzymatic digestion or simply by cell autolysis followed

by subsequent isolation of the desired product. The polypeptide can be separated from the cell mass by various means. In one such method the cells are disrupted by the protease ficin and subjected to ultrafiltration. The polypeptide is subsequently precipitated with an organic solvent such as methanol or acetone. The polypeptide can also be separated from the cell mass by suspending the microorganism in a brine solution sufficient to partition the polypeptide into the brine solution (for example 20% (w/v) NaCl). It is suggested that the brine solution creates osmotic pressure sufficient enough to partition a polypeptide into the brine solution. In general the same methods heretofore employed to liberate and produce solutions of other intracellular enzymes can be employed.

The polypeptide isolated from microbial cells is generally purified by conventional precipitation and chromatographic methods. Such methods include amongst others methanol, ethanol, acetone and ammonium sulfate precipitation and ion exchange and hydroxy apatite chromatography.

As already stated hereinabove, the excellent stability of the hydrophobin complexes, especially against SDS and other surfactants, may be used with advantage in isolating the hydrophobins both from the cells in which they are produced, from a culture medium in which they have been secreted, which may also contain cell fragments and debris.

The invention will now be illustrated by means of the following non-limiting examples.

Example 1

1.1 Isolation of highly expressed fruit body specific genes of *A. bisporus*

All techniques used for the manipulation and analysis of nucleic acid materials were performed essentially as described in Sambrook et al. (1989), except where indicated otherwise.

1.1.1. Isolation of total RNA from vegetatively growing mycelium of

Horst[®] U1

Mycelium of Horst[®] U1 was grown on agar plates containing DT80 glucose medium (Sonnenberg et al., 1988) covered with a cellophane sheet. After 14 days of incubation at 24°C, colonies were collected, cut up in a Waring blender and used to inoculate Fernbach flasks each containing 200 ml DT80 glucose medium.

The cultures were allowed to grow stationary for 14 days at 24°C and were harvested by filtration over nylon gauze. The mycelium was immediately frozen in liquid nitrogen and stored at -70°C. Total RNA was isolated from the mycelium by a standard method which uses guanidinium thiocyanate essentially as described by Sambrook et al. (1989).

1.1.2. Isolation of total RNA from primordia and fruit bodies of sizes smaller than 5 mm (pins) of Horst[®] U1 and construction of a mixed cDNA library of primordia and pins.

Mycelium of *Agaricus bisporus* Horst[®] U1 was allowed to colonize compost using standard cultivation conditions. A top layer (casing) was added to induce fruit body formation. The primordia and pins were picked from the first flush, immediately frozen in liquid nitrogen and stored at -70°C. The deep frozen tissue was grinded in a precooled Waring blender and total RNA was isolated from this tissue using a standard method (section 1.1.1.). The obtained total RNA was subsequently used as source for the isolation of polyA⁺ RNA by a standard method (Sambrook et al., (1989)). The polyA⁺ RNA was subsequently used as a source for the generation of complement DNA (cDNA) using a λ -ZAP-cDNA synthesis kit (Stratagene, La Jolla). In this way a library was obtained of 1.5×10^5 pfu.

1.1.3. Isolation of total RNA from stage 7 fruit bodies of Horst[®] U1 A. *bisporus* stage 7 mushrooms were picked from the first flush, immediately frozen in liquid nitrogen and stored at -70°C. The deep frozen tissue was grinded in a precooled Waring blender and total RNA was isolated from this tissue using a standard method (section 1.1.1.) (Sambrook et al., (1989)).

1.1.4. Screening of the *Agaricus bisporus* mixed primordia - fruit

body cDNA library for highly expressed genes.

The *A. bisporus* λ -ZAP-cDNA library was used to generate 200 random cDNA clones using a standard protocol. The insert of each individual clone was amplified using polymerase chain reaction (PCR) amplification using a Perkin-Elmer Cetus Thermal Cycler and a program of 20 thermal cycles, each consisting incubations of 1 min. at 94°C, 1 min. at 62°C and 1 min. at 72°C preceded by an incubation of 4 min. 95°C and followed by an incubation of 5 min. at 72°C. The oligonucleotides used were standard SK and T7 primers which are able to anneal directly adjacent to the cDNA insert on either side. The reactions were performed in the presence of 200 μ M each of dATP, dCTP, dTTP and dGTP, 1 units Taq polymerase (Boehringer Mannheim), 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin and 50 pmol of each primer in a total volume of 25 μ l, using plasmid DNA obtained from an *A. bisporus* cDNA clone as template.

The DNA templates were isolated by boiling a part of an *Escherichia coli* colony bearing the desired cDNA containing plasmid in H₂O for 5 min. followed by removal of the cell debris by centrifugation. Each individual PCR product was radioactively labeled using the method of Feinberg and Vogelstein (1983) and used as a probe in Northern analyses of 5 μ g and 0.05 μ g of total RNA isolated from mycelium (section 1.1.1.), 5 μ g and 0.05 μ g of total RNA isolated from a mixture of primordia and small fruit bodies (section 1.1.2) and 5 μ g of total RNA isolated from stage 7 fruit bodies (section 1.1.3) spotted on Hybond N membrane (Amersham). Hybridization was performed o/n in a standard solution of 6x SSC, 0.5% SDS and 5x Denhardt's solution and 100 μ g/ml single stranded herring sperm DNA at 65°C followed by stringent standard wash procedures. Two complementary DNA inserts which gave very high hybridizing signals with total RNA isolated from a mixture of pins and primordia and stage 7 mushrooms but gave low or not detectable hybridizing signals with total RNA isolated from vegetatively growing mycelium were analyzed further by sequencing and Southern analysis. The matching genes were called hypA and hypB after the hydrophobic properties of the inferred polypeptide sequence they encode. The gene encoding HYP A (hypA) is highly expressed in both pins and stage 7 fruit bodies but appears

to be not expressed in vegetatively growing mycelium. The gene encoding HYPB (hypB) is more expressed in pins than in stage 7 fruit bodies but appears to be not expressed in vegetatively growing mycelium.

1.2. Construction and screening of a genomic library of *Agaricus bisporus* strain 39 for the genes encoding hypA, hypB and hypC.

A genomic library of *A. bisporus* strain 39, one of the homokaryotic constituents of Horst [®] U1 was obtained by growing mycelium of strain 39 in Fernbach flasks on DT80 glucose medium as described in section 1.1.1. Mycelium was harvested and genomic DNA was isolated from this mycelium using the method of De Graaff et al. (1988). Partial MboI digested DNA was size fractionated on a sucrose gradient and 8 to 21 kb fragments were cloned into the BamHI site of λ EMBL4 (Sambrook et al., 1989). After packaging the obtained recombinant phages were used to infect competent LE392 cells for amplification. A genomic library of 2×10^{10} plaques was obtained. The genomic library was screened with two cDNAs of genes (hypA and hypB). The cDNA probes were labeled according to method of Feinberg and Vogelstein and approximately 25×10^3 plaques were screened in duplo with the cDNA probes according to standard methods (Sambrook et al., 1989) using *Escherichia coli* LE392 as plating bacteria. The total length of the inserts contained within the analyzed plaques is equivalent to about 10 times the size of the *Agaricus bisporus* genome (Sonnenberg et al., 1991). Hybridization and wash conditions were as described in in section 1.1.4. Plaques which scored positive for hybridization to the probes on duplicate sets of filters were purified according to standard methods and of each gene and according to standard methods. For each gene was DNA isolated of four of those positive plaques.

1.2.1 Cloning and characterization of the *A. bisporus* hypA gene and hypC gene.

1.2.1.1. Physical mapping of four overlapping λ clones containing the *Agaricus bisporus* hypA gene and hypC gene.

The inserts of four positive clones were analyzed by Southern hybridization of single and combined digestion with the restriction enzymes EcoRI, BamHI, HindIII, SalI and BglII using the hypA cDNA (SEQ ID No: 1.) as a probe.

Combination of the resulting data led to the identification of 1.5 kb EcoRI fragment, an adjacent 1.8 kb EcoRI-HindIII fragment and an overlapping 4.2 kb BglII fragment which all hybridized with the cDNA

of the *Agaricus bisporus* hypA gene (Fig. 1).

1.2.1.2. Sequencing of the *Agaricus bisporus* hypA and hypC genes.

The 1.5 kb EcoRI fragment from the positive λ clones was subcloned in the EcoRI site of pUC19 (Yannish-Perron et al., 1985) yielding pIM3103 (Fig. 1.). The 1.8 kb EcoRI-HindIII fragment from the positive λ clones was subcloned between the EcoRI and HindIII sites of pUC19 yielding pIM3102 (Fig. 1.). The 4.2 kb BglII fragment from the positive λ clones was subcloned between in the BamHI site of pUC19 yielding pIM3104 (Fig. 1.). An *Escherichia coli* DH5 α strain containing this plasmid (CBS 403.94) was deposited at CBS, Baarn, the Netherlands on May 19, 1995. The sequence of the 1.5 kb EcoRI and the 1.8 kb EcoRI-HindIII fragment fragment was determined by sequencing the entire insert of pIM3102 and pIM3103 and derived subclones in both directions according to the method of Sanger (1977) using standard and dedicated synthetic primers. Additional sequence information was obtained from sequencing the insert of pIM3104 and derived subclones in both directions using standard and dedicated synthetic primers. (Fig. 3, sequence listing no. 2.)

1.2.1.3. Identification of the open reading frame of the *Agaricus bisporus* hypA gene.

In order to identify the exact size and location of the intervening sequences in the *A. bisporus* hypA gene the cDNA insert of pIM3100 was completely sequenced in both directions using standard methods as described in section 1.2.1.2. Comparison of the obtained cDNA sequence with the genomic DNA sequence of the *A. bisporus* hypA gene unambiguously identified the position and size of the 3 introns. (Fig. 2 and 3) (SEQ ID NO: 1 and SEQ ID NO: 2).

1.2.1.4. Identification of the open reading frame of the *Agaricus bisporus* hypC gene.

Comparison of the DNA sequences of the insert of pIM3103 with the cDNA (pIM3100) of the hypA gene revealed the presence of an homologous sequence adjacent to the hypA gene (hypC). The location of the intervening sequences in the *A. bisporus* hypC gene correlates with the previous identified 3 introns in the hypA gene. (Fig. 2 and

3.)(SEQ ID NO: 1 and SEQ ID NO: 2.)

1.2.1.5 Identification of the intergenic region of hypA and hypC.

In order to determine the orientation and exact location of hypA and hypC on the 4.2 kb BglII fragment the relevant part of the insert of pIM3104 was sequenced using dedicated synthetic primers. Both genes appeared to have the same orientation on pIM3104, hypC being positioned downstream of hypA. Sequence analysis also showed that the 1.5 EcoRI fragment and the 1.8 EcoRI- HindIII fragment both have the same intergenic EcoRI restriction enzyme site, the length of the intergenic region of hypA and hypC being about 2.3 kb (Fig. 3, sequence listing no. 2.).

1.2.2. Cloning and characterization of the *A. bisporus* hypB gene

1.2.2.1. Physical mapping of four overlapping λ clones containing the *Agaricus bisporus* hypB gene and sequencing of the *Agaricus bisporus* hypB gene.

The inserts of four positive clones obtained by screening the strain 39 genomic library for hybridizing λ clones containing the *Agaricus bisporus* hypB gene using the hypB cDNA clone as probe (see section 1.2), were analyzed by Southern hybridization of single and combined digestion with the restriction enzymes EcoRI, BamHI, HindIII, Sall and BglII using the hypB cDNA (SEQ ID No: 3.) as a probe. Combination of the resulting data led to the identification of 4.7 kb EcoRI fragment comprising a 1.8 kb BamHI-EcoRI fragment and an adjacent 2.9 kb bamHI-EcoRI fragment which both hybridized with the cDNA of the *Agaricus bisporus* hypB gene (Fig. 4). The 4.7 kb EcoRI fragment from the positive λ clones was subcloned in the EcoRI site of pUC19 yielding pIM3108 (Fig. 11.) An *Escherichia coli* DH5a strain containing this plasmid (CBS 404.95) was deposited at CBS, Baarn, the Netherlands on May 19, 1995. The 1.8 kb BamHI- EcoRI fragment and 2.9 kb BamHI-EcoRI fragment from the positive λ clones were subcloned between the BamHI and EcoRI sites of pUC19 yielding pIM3106 and pIM3107 (Fig. 4). The sequence of the relevant parts of the 1.8 kb BamHI-EcoRI and the 2.9 kb BamHI-EcoRI fragment was determined by sequencing parts of the inserts of pIM3106 and pIM3107

and derived subclones in both directions as described in section 1.2.1.2. using standard and dedicated synthetic primers (Fig. 6, sequence listing no. 4). Additional sequence information was obtained from partial sequencing of the relevant parts of the insert of pIM3108 using dedicated synthetic primers.

1.2.2.2. Identification of the open reading frame of the *Agaricus bisporus* hypB gene.

The exact size and location of the intervening sequences in the *A. bisporus* hypB gene were identified by sequencing the cDNA insert of pIM3101 in both directions using standard methods as described in section 1.2.1.2. and comparison of the obtained cDNA sequence with the genomic DNA sequence of the *A. bisporus* hypB gene. In this way we could unambiguously identify the position and size of the two introns. (Fig. 5 and 6) (SEQ ID NO: 3 and SEQ ID NO: 4.)

1.2.2.3 Cloning and characterization of the hypD gene of *Agaricus bisporus*

Genomic DNA of the homokaryotic strains 39 and 97 was analyzed by Southern hybridization of single and combined digestion with the restriction enzymes EcoRI, BamHI, HindIII, SalI and BglII using the hypB cDNA (SEQ ID No: 3.) as a probe. Comparison of the hybridization pattern of this blot with the genomic DNA to the hybridization pattern of the Southern blot with the positive hypB λ clones (section 1.2.2.1) showed one or two extra hybridizing bands in each lane of the blot with the genomic DNA, suggesting the possible existence of a second hypB like gene, hypD, in *Agaricus bisporus*. This was confirmed by CHEF-analysis (Sonnenberg et al., 1991) in which blots containing DNA of strains 39 and 97 showed two hybridizing chromosomal bands (chromosomes 1 and 12) of equal strength using the hypB cDNA clone as a probe (Fig. 12B). When a 1 kb BamHI- EcoRI fragment of hypB phage 1, located 2 kb upstream of hypB, was used as a hypB specific probe, hybridization was only found with chromosome 12 (Fig 12A), hereby locating the hypB gene on chromosome 12 and hypD on chromosome 1. Southern analysis of ten positive hypB λ clones double digested with EcoRI and BamHI showed,

that only the clones 1, 3 and 7 hybridized when the fragment located 2 kb upstream of hypB was used as a probe. By Southern-analysis of clone 10 using the hypB cDNA clone as a probe the hypD gene was located on a 3.5 kb EcoRI-BamHI fragment. This 3.5 kb EcoRI-BamHI fragment will be chosen for cloning experiments to identify the open reading frame of

the *Agaricus bisporus* hypD gene. A 1.2 kb EcoRI-BamHI fragment of clone 10 which did not hybridize with the hypB cDNA probe was used as a hypD specific probe in a CHEF-analysis of strain 39 and 97. In this hybridization experiment the hypD specific probe, as expected, only showed hybridization to chromosome 1 (Fig. 12C), confirming the location of hypD on this chromosome (see above).

Example 2

Overproduction of *Agaricus bisporus* HYPA and HYPB in *Agaricus bisporus* controlled by regulatory elements of *Agaricus bisporus* hypA or hypB genes.

It is now possible to transform a diverse array of eucaryotic cells including *A. bisporus* cells with exogenic DNA, thereby selectively incorporating individual genes determining economically important traits into the genetic background of an individual line of *A. bisporus*.

At the Fifth International Mycological Congress in Vancouver which was held from August 14-21, 1994, two groups (Van De Rhee, Graca and Mooibroek and Schuren and Wessels) independently reported transformation of *A. bisporus* cells. These transformation systems can be used to transform *A. bisporus* cells with the complete *A. bisporus* hypA and hypB genes using their own promoters or other available strong promoters of *A. bisporus*, such as the available promoter of the constitutive and highly expressed glyceraldehyde-3-phosphate dehydrogenase (gpd) gene (Harmsen et al., 1992).

Example 3

Production of heterologous proteins in *Agaricus bisporus* controlled by regulatory elements of *Agaricus bisporus* hypA or hypB genes

5 The *A. bisporus* hypA or the hypB regulatory elements can also be used for expression of other *A. bisporus* proteins or heterologous proteins in *A. bisporus* fruit bodies i.e. proteins that influence the morphology and/or yield and nutrition value of the basidocarp, proteins that improve the quality of the fruit body, proteins that
10 enhance the flavour or proteins that improve the shelf life of the crop. Using the regulatory regions of either the hypA or hypB genes for the production of (heterologous) proteins), we are able to regulate the expression of the desired protein to defined stages of development. The *A. bisporus* hypB gene is highly expressed early
15 during development of the basidiocarp, while the hypA gene is highly expressed during fruit body elongation and maturation. When the desired protein is preferably expressed during fruit body development, the hypB promoter will be used, however when the desired protein is preferably expressed during fruit body elongation
20 or maturation the most suitable promoter will be the hypA promoter. The fusion constructs replacing the promoters of the gene encoding the desired protein by the selected *A. bisporus* hyp promoter can be made using standard cloning techniques, PCR or synthetic oligonucleotides.

Example 4

Production of *Agaricus bisporus* HYPA and HYPB in *Aspergilli* controlled by other regulatory elements than those derived from the
30 *Agaricus bisporus* hypA or hypB genes.

For the efficient production of *A. bisporus* HYPA and HYPB in *Aspergillus* species such as *Aspergillus niger* CBS 120.49 or *Aspergillus niger* var. *awamori* CBS 115.51 or related species
35 functional fusions can be made using regulatory sequences of highly expressed *Aspergillus* genes such as the *gpd* promoter and terminator and the *A. bisporus* hypA or the hypB gene. The fusions can be made

using PCR or synthetic oligonucleotides. Such constructions can be integrated in the *A. bisporus* genome in single or multi copies using standard *Aspergillus* cotransformation techniques. As an acceptor strain in transformation experiments *Aspergillus niger* NW128 (*cspA1*, *goxC17*, *pyrA6*, *nicA1*) can be used. The *cspA1* mutation (short conidiophores) facilitates the handling of the strain on plates, the *gox* mutation (no production of glucose oxidase) facilitates the evaluation of HYPA or HYPB production during a fermentation experiment and the production of a pure HYPA or HYPB preparation, the *pyrA1* mutation (requirement for uridine) can be utilized for the introduction of multiple copies of the *hypA* and *hypB* genes, and the *nicA1* mutation (requirement for nicotinamide) facilitates the biologically contained handling of the strain (Witteveen et al., 1990). *Aspergillus niger* strain NW128 can be co-transformed with mixtures of two different DNA fragments in various ratios using standard techniques (e.g. Goosen et al., 1987). One of these fragments will be the 3.8 kb *XbaI* fragment of *Aspergillus niger* N400, comprising the entire *pyrA* gene and functional promoter (Goosen et al., 1987). The other fragment will be the *hyp* fusion gene. If secretion is desired, DNA sequences encoding functional signal sequences derived from *A. bisporus* *hypA* or *hypB* genes encoding proteins that are efficiently secreted can be used. Alternatively functional signal sequences optimized for *A. niger* can be added in a functional fusion before the region encoding the mature HYP protein mentioned above using PCR or synthetic oligonucleotides. The levels of expression of heterologous proteins can be further improved by adjustment of the codon usage to the preferred codon usage of *A. niger*.

Example 5

Production of *Agaricus bisporus* HYPA and HYPB in yeast

For the production of *A. bisporus* HYPA and HYPB in yeasts, vectors can be constructed in which sequences encoding the mature *A. bisporus* HYPA and HYPB proteins are placed under the control of

known yeast promoters. Since yeasts cannot recognize all the introns of the *A. bisporus* hypA and hypB genes, the constructs should be made from the complete cDNAs of hypA and hypB (plasmids pIM3100 and pIM3101) which can be used as starting material for the construction of functional fusions between yeast promoters and functional sequences such as yeast translational start signals and part of the *A. bisporus* cDNAs of hypA or hypB encoding the mature HYPA or HYPB. The fusions can be made using PCR or synthetic oligonucleotides. Such constructions can be incorporated in autonomously replicating yeast vectors or can be integrated in the yeast genome in single or multi copies using the appropriate yeast vectors. If secretion is desired, DNA sequences encoding functional yeast signal sequences derived from yeast genes encoding proteins that are efficiently secreted can be added in a functional fusion before the region encoding the mature HYPA or HYPB using PCR or synthetic oligonucleotides. The levels of expression can be further improved by adjustment of the codon usage of the *A. bisporus* hypA and hypB genes according to the codon preferences known for yeasts.

In another aspect the invention relates to regulatory regions of the hydrophobin genes mentioned hereinabove.

More in particular, this aspect of the invention is related to the regulatory regions and sequences of the hydrophobin genes of *Agaricus bisporus*, more in particular to the promoters of the hyp A, hyp B, hyp C and hyp D genes of *Agaricus bisporus*.

In the field of biotechnology, there is a constant search for new regulatory sequences that can be used for the expression of desired homologous or heterologous genes for the production of desired polypeptides or other genetically encoded products.

This is especially true for organisms in which the lack of appropriate regulatory sequences has stifled the further progress of recombinant DNA-technology, such as fungi.

A very desirable organism for the production of desired polypeptides would be the common mushroom *Agaricus bisporus*, because it is a food grade organism of GRAS status, the fruit bodies of which are edible. Also, a lot is known about the cultivation,

production and the processing of mushrooms, knowledge that could be used directly for the production of desired polypeptides in these organisms. Finally, mushrooms can be cultivated cheaply and economically on a large scale, even on industrial scale.

5 It is therefore an object of the present invention to provide regulatory regions, regulatory sequences and/or promoters that can be used for the expression of homologous or heterologous genes in fungi, especially edible fungi, in particular fungi from the genus *Agaricus* and more in particular in *Agaricus bisporus*.

10 It has now been found that the regulatory regions of the hydrophobin genes of edible fungi, especially the regulatory regions of said genes which are activated in vivo during the emergence or formation of the fruit body, and more specifically, the regulatory regions of the hydrophobin genes of fungi from the genus *Agaricus*
15 can with advantage be used for expression of homologous or heterologous genes. The invention therefore relates to said regulatory regions.

 More in particular, the invention relates to the regulatory regions and sequences of the hydrophobin genes of *Agaricus bisporus*,
20 more in particular to the promoters of the hyp A, hyp B, hyp C and hyp D genes of *Agaricus bisporus*.

 It is to be understood that the regulatory regions of the invention will usually comprise at least a promoter, optionally an operon.

25 These regulatory regions, which have now been identified and characterized for the first time, are in vivo activated only during the emergence of the fruit bodies. Therefore, the genes controlled by these regulatory regions are only expressed during the formation of or in the fruit bodies, so that the desired polypeptides for
30 which said genes encode are (only) secreted into the fruit body, which can then be harvested and processed for the isolation of the desired polypeptide, which process is generally easy to perform with mushrooms. Moreover, as only the fruit body is harvested, the rest of the fungal organism will stay alive and intact to form new fruit
35 bodies producing the desired polypeptides.

 Also, during the formation of the fruit body, the regulatory regions of the invention provide a very abundant expression of the product

encoded the gene which they control. Genes controlled by said regulatory sequences usually account for more than about 4-6% of the mRNA present in the fruit bodies.

5 These advantages make the regulatory regions of the invention very suitable for the production of polypeptides in mushrooms.

The regulatory regions of the invention can be used to express any desired gene or nucleotide sequence, both homologous or heterologous, in a suitable fungus. Preferably, this fungus is a food grade fungus, more preferably an edible fungus, and most preferred a fungus with GRAS status. The fungus used as expression host is also preferably the fungus from which the regulatory region was originally derived.

15 However, the regulatory regions of the invention can also be used as heterologous regulatory systems in other organisms, especially in other fungi, in which case they can provide a more abundant expression of the controlled genes, compared to the homologous regulatory regions of the fungal expression host.

20 In a preferred embodiment, the invention relates to the promoters of the hyp A, hyp B, hyp C and hyp D genes of *Agaricus bisporus*. The sequence listings of the regulatory regions/promoters of the *Agaricus bisporus* hyp A, hyp B and hyp C genes are shown in the sequence ID's for these respective genes as mentioned hereinabove.

25 The hyp A promoter has a TATA-box, shown in the sequence listing of pIM 3104. The hyp A promoter also contains an essential element of the enhancer more than 290 bp upstream of the translation start. In hyp C, the TATA box is positioned about 130 bp before the translation start codon. The TATA-box of the hypB promoter is shown in the sequence ID of pIM 3104.

30 The regulatory regions of the invention that control the hydrophobin genes are usually positioned so as to operably control the expression of said hydrophobin genes, as will be clear to person skilled in the art. In general, this means that the regulatory regions will be found upstream of the structural genes coding for the hydrophobins.

35 Homologous or heterologous genes, for which it is desired that they are controlled by the regulatory regions of the invention,

can be put under the control of the regulatory regions by operably linking them to said regulatory regions, as will be clear to a person skilled in the art. This can for instance be done by inserting said homologous or heterologous gene in a recombinant DNA fragment containing said regulatory region, in a manner known per se, and then introducing said recombinant DNA fragment containing the regulatory region and the nucleotide sequence encoding for the polypeptide or protein into the fungus that is used as the expression host. As plasmids are generally not maintained in fungi, introducing this recombinant DNA material is preferably done by incorporation in the genomic DNA of the fungus.

Genetic manipulation and transformation of fungi, especially edible fungi, has been described in the state of the art, for instance in the references mentioned in this application, that are incorporated herein by reference, and all known techniques can be used. Furthermore, other recombinant DNA and/or transformation techniques known to a person skilled in the art can be used, as long as they provide the desired result, as will be clear to a person skilled in the art. The invention is therefore not limited to any specific method of isolating/identifying the regulatory regions of the invention, for operably linking said regulatory regions to homologous or heterologous genes to be expressed, or for transforming a selected expression host with the genetic material thus obtained.

The genes can then be expressed by inducing the regulatory gene with a suitable inducer. However, according to the preferred embodiment, in which the regulatory region is activated during the emergence and/or formation of the fruit body, the preferred way of expressing the genes coding for the desired polypeptide or proteins is simply by cultivation of the fungi, whereby expression will take place during formation of the fruit body, and the proteins will simply be secreted in or into the fruit body. As described hereinabove, the desired proteins or polypeptides can then simply be obtained by harvesting fruit bodies and isolating the polypeptides or proteins in a manner known per se.

It is to be understood that the desired polypeptides or proteins can also be expressed as a ripening form as defined

hereinabove.

It is to be understood that the invention further comprises all homologous and genetic variants of the regulatory regions of the invention as defined hereinabove, so long as they are still able to control the expression of a gene operably linked thereto. As such, these homologous or genetic variants can provide for decreased, the same or increased expression of the gene compared to the original regulatory sequence from which it was derived. These homologous and genetic variants may also have other advantageous properties, such as thermo-inducibility, "runaway" expression or continuous expression of the controlled genes. Furthermore, the term genetic variant explicitly includes regulatory regions which are only a part of the original regulatory region from which it was derived, i.e. obtained by deletion of one or more nucleotides, both at the 5' or 3' termini or somewhere inbetween, so long as these parts of the original regulatory regions still control the expression of a structural gene operably linked thereto.

The hyp promoters can be used to express in fruit bodies for instance in sense or antisense direction the clones *A. bisporus* mannitol dehydrogenase gene or glucose-6-phosphate dehydrogenase gene (Wood et al., 1991), the *A. bisporus* methallothionein genes (Nishiyama et al., 1991) or the cloned putative tyrosinase genes. In addition the promoters can be used to express e.g. a coat protein of a dsRNA virus and generate in this way resistance through cross-protection (Harmsen et al., 1989, Harsem et al., 1991). These hyp promoters can also be used to express heterologous proteins *A. bisporus* in fruit bodies such as hormones for instance adrenocorticotropin, melanocyte stimulating hormone, urogastrone or insulin or growth factors such as epidermal growth factor insulin-like growth factor interleukin, such as interleukin-1 or -2, interferons such as human interferon and proteinase inhibitors such as α 1-antitrypsin and immunoglobulins such as the light or heavy chains of immunoglobulin D, E or G.

References cited in the application:

- 5 Yanish-Perron C., Vieira J. and J. Messing. (1985) Improved phage M13 cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* 33: 103-119.
- 10 Sanger F., Nicklen S. and A.R. Coulson. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci., U.S.A.* 74: 5463-5467.
- Maniatis T., Fritsch E.F. and J. Sambrook. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
- 15 Gietz R.D. and A. Sugino. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base-pair restriction sites. *Gene* 74: 527-534.
- 20 Harmsen M.C., Schuren F.H.J., Moukha S.M., Van Zuilen C.M., Punt P.J. and J.G.H. Wessels (1992). Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. *Curr. Genet.* 22: 447-454.
- 25 Sonnenberg A.S., Wessels J.G. and L.J. van Griensven. (1988). An efficient protoplasting/regeneration system for *Agaricus bisporus* and *Agaricus bitorquis*. *Curr. Microbiol.* 17: 285-291.
- 30 Sonnenberg A.S., Den Hollander K., Van de Munckhof A.P.J. and L.J.L.D. van Griensven (1991). Chromosomes separation and assignment of DNA probes in *Agaricus bisporus*. In: Van Griensven L.J.L.D. (ed) *Genetics and breeding of Agaricus*. Pudoc, Wageningen, pp. 57-61.
- 35 Goosen T., Bloemheuvel G., Gysler C., De Bie D.A., Van den Broek H.W.J. and K. Swart. (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate decarboxylase gene. *Curr. Genet.* 11: 499-503.

- Witteveen C.F., Van de Vondervoort P.J.I., Van den Broeck H.C., Van Engelenburg F.A.C., De Graaff L.H., Hillebrand M.H.B.C., Schaap P.J. and J. Visser. (1993) Induction of glucose oxidase, catalase, lactonase in *Aspergillus niger*. Curr. Genet. (199?)
- 5 Van De Rhee M., Graça P. and H. Mooibroek. (1994) Transformation of common mushroom, *Agaricus bisporus*. Abstract at the Fifth International Mycological Congress in Vancouver which was held from August 14-21, p.228.
- 10 Schuren F.H.J. and J.G.H. Wessels. (1994) The use of *GPD* sequences of *Agaricus bisporus* in transformation experiments. Abstract at the Fifth International Mycological Congress in Vancouver which was held from August 14-21, p.194.
- 15 Feinberg A.P. and Vogelstein B. 1983. A technique for radiolabelling DNA restriction fragments to high specific activity. Anal. Biochem. 132: 6-13.
- 20 Sambrook J., Fritsch E.F. and T. Maniatis. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
- 25 Nishiyama Y., Nakayama S., Okada Y., Min K-S., Onosaka S. and Tanaka K. (1990) Chem. Pharm. Bull. 38: 2112-2117.
- Harmsen M.C., Tolner B., Kram A., Go S.J., De Haan A. and Wessels J.G.H. (1991) Curr. Genet. 20: 137-144.
- 30 Harmsen M.C., Van Griensven L.J.L.D. and Wessels J.G.H (1989) J. Gen. Virol. 70: 1613-1616.
- 35 Wood D.A., Claydon N., Burton K.S., Matcham S.E., Allan M., Perry C., Thurston C.F., Raguz S. and E. Yague (1991) In: Science and cultivation of edible fungi, Maher (ed.) Balkema Rotterdam, ISBN 9054100214, pp. 43-50.

CLAIMS

1. Hydrophobin, or a protein or polypeptide essentially corresponding thereto, derived from an edible fungus.
5
2. Hydrophobin, or a protein or polypeptide essentially corresponding thereto, derived from an edible fungus with GRAS status.
- 10 3. Hydrophobin according to claim 1 or 2, or a protein or polypeptide essentially corresponding thereto, from a fungus from the genus *Agaricus*, preferably *Agaricus bisporus*.
- 15 4. Hydrophobin according to any of the claims 1-3, or a protein or polypeptide essentially corresponding thereto, derived from an edible fruit body.
- 20 5. Gene, nucleotide sequence or DNA-fragment, encoding a ripening form of a hydrophobin, or a protein or polypeptide essentially corresponding thereto, according to claims 1-3, as well as genetic variants and homologues thereof.
- 25 6. Nucleotide sequence or DNA-fragment according to claim 3, derived from the hyp A, hyp B, hyp C or Hyp D genes of *Agaricus bisporus*, as well as genetic variants and homologues thereof.
- 30 7. Nucleotide sequence according to claim 6, chosen from sequence listing ID no 1, sequence listing ID no 2 or sequence listing ID no 3, as well as genetic variants and homologues thereof.
- 35 8. A recombinant DNA material comprising at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus, wherein the nucleotide sequence encoding the polypeptide is selected from the group consisting of
 - a) at least a part of a nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus;
 - b) a genetic variant of a nucleotide sequence according to a);

- c) a nucleotide sequence capable of hybridizing to either of the nucleotide sequences a) or b).

5 9. A recombinant DNA material according to claim 8 wherein the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus is selected from the group consisting of

- a) at least a part of a gene, nucleotide sequence or DNA-fragment according to any of the claims 5-7,
b) a genetic variant of the part of a nucleotide sequence
10 according to a);
c) a nucleotide sequence capable of hybridizing to either of the parts of a nucleotide sequence according to a) or b).

15 10. A recombinant DNA material according to any of the claim 8-9, wherein the nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus is operably linked to at least one regulatory region capable of directing the expression of said nucleotide sequence.

20 11. A recombinant DNA material according to claim 10 wherein the regulatory region is derived from another strain of organism than the organism from which the nucleotide sequence is derived.

25 12. A recombinant DNA material according to any of the claims 8-11, being a cloning vehicle, preferably a plasmid.

13. A recombinant DNA material according to claim 12, being plasmid pIM 3100, pIM 3101, pIM 3102, pIM 3103, pIM 3104 or pIM 3106.

30 14. A recombinant DNA material according to any of the claims 8-13, wherein said recombinant DNA material comprises at least one selection marker gene.

15. A cell comprising at least a part of recombinant DNA material according to any of the claims 8-14.

35 16. A cell according to claim 15, said cell being capable of expression, preferably being capable of overexpression of a

nucleotide sequence encoding a ripening form of a hydrophobin of an edible fungus as encoded by said recombinant DNA material.

5 17. A cell according to claim 15 or 16, wherein said cell is a food grade cell.

10 18. Cell according to claim 15, being an Escherichia coli DH5 α strain containing plasmid pIM 3104 as deposited at the Centraal Bureau voor Schimmelcultures (CBS) Baarn, the Netherlands on May 19, 1995 under accession number CBS 403.95, or an Escherichia coli DH5 α strain containing plasmid pIM 3106 as deposited at the Centraal Bureau voor Schimmelcultures (CBS) Baarn, the Netherlands on May 19, 1995 under accession number CBS 404.95

15 19. A process for producing a ripening form of a Hydrophobin, or a protein or polypeptide essentially corresponding thereto, derived from an edible fungus,
20 comprising expression of a gene, gene sequence or a DNA-fragment according to claims 5-7 and/or
comprising the culture of a cell according to any of the claims 15-17 and optionally isolation of the resulting ripening form of a hydrophobin of an edible fungus.

25 20. Compositions, especially foodstuffs, pharmaceutical preparations and/or personal care preparations, comprising
a hydrophobin according to any of the claims 1-4, or a peptide or protein essentially corresponding thereto.

30 21. Use of a hydrophobin according to any of the claims 1-4, or a peptide or protein essentially corresponding thereto, as an emulgator or surfactant, or as a preservative for foodstuffs, or in the field of environmental technology.

35 22. Regulatory region of a hydrophobin gene from an edible fungus.

23. Regulatory region according to claim 22, said regulatory region

being activated during the emergence and/or formation of the fruit body of the edible fungus.

5 24. Regulatory region according to claim 21 or 22, derived from a fungus with GRAS-status, especially a fungus from the genus Agaricus, preferably from Agaricus bisporus.

10 25. Regulatory region according to any of the claims 21-24, of a fungus of the genus Agaricus, especially Agaricus bisporus.

26. Regulatory region according to claim 25, comprising one or more promoters of the hyp A, hyp B, hyp C and hyp D genes of Agaricus bisporus.

15 27. Regulatory region according to claim 25 or 26, having a sequence chosen from the relevant regulatory regions from sequence listings ID NO 1-3.

20 28. Recombinant DNA-material, comprising a regulatory region according to claims 22-27.

25 29. Recombinant DNA-material according to claim 28, further comprising a homologous or heterologous gene encoding a ripening form of a desired polypeptide, other than the hydrophobin natively controlled by said regulatory region, operably linked to said regulatory region.

30 30. Fungus, especially an edible fungus, in particular a fungus with GRAS-status, comprising a recombinant DNA-material according to claim 29.

31. Fungus according to claim 30, of the genus Agaricus, especially Agaricus bisporus.

35 32. Fungus according to claim 30 or 31, wherein the recombinant DNA-material is incorporated into the genomic DNA.

33. Method for expressing one or more homologous or heterologous genes in a fungus, preferably an edible fungus, more preferably a fungus with GRAS-status, in particular a fungus of the genus Agaricus, comprising

- 5 - operably bringing said gene(s) under the control of a regulatory region according to claims 22-29
 - bringing said gene(s) to controlled expression.

10 34. Method according to claim 33, in which the homologous or heterologous genes are expressed in the species or strain of Agaricus from which the regulatory region according to claim 22-29 has been derived.

15 35. Method for producing a ripening form of a desired polypeptide in fungi of the genus Agaricus, comprising bringing one or more genes coding for said polypeptide to expression according to claims 33-34 or, and isolating the ripening form of the expressed polypeptide.

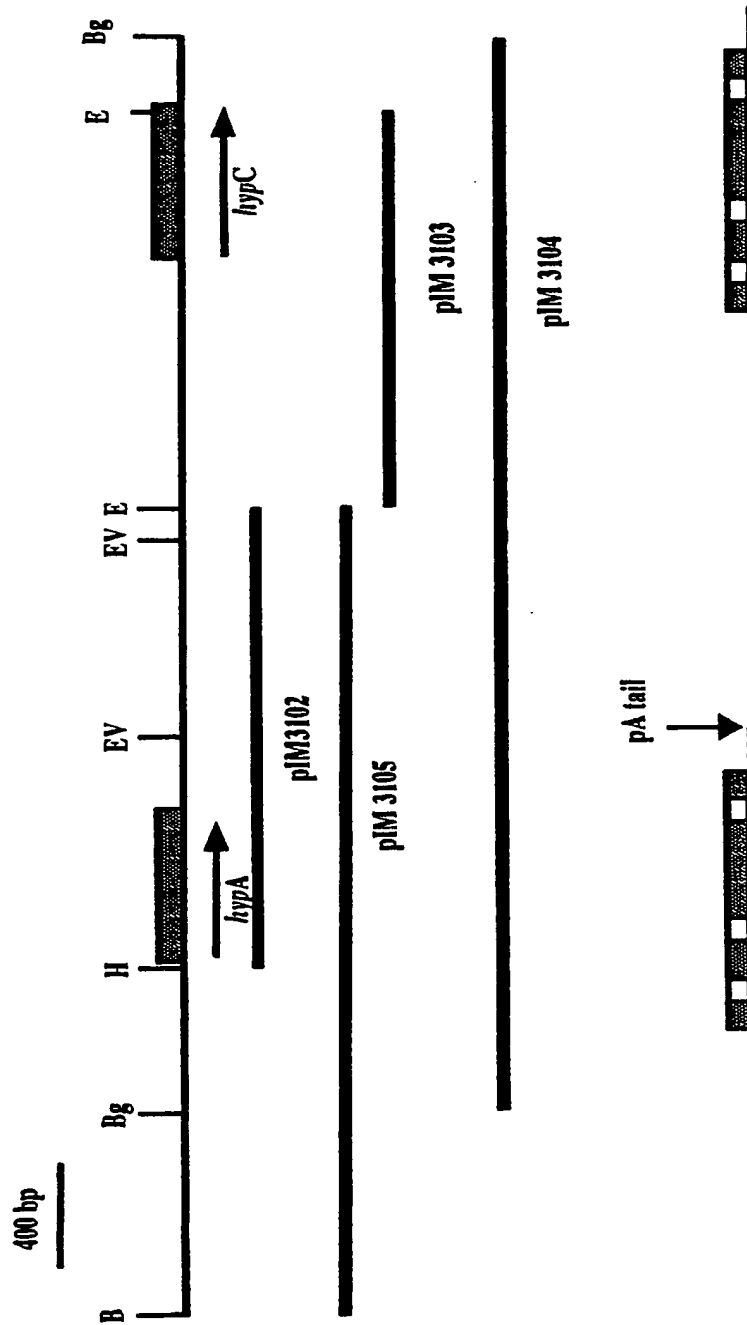
20 36. Method for producing a ripening form of a desired polypeptide according to claim 35, comprising
 - cultivating a fungus according to any of the claims 30-32,
 - harvesting the fruit bodies, and
 - isolating the ripening form of the desired polypeptide from the harvested fruit body.

25 37. Use of fruit bodies of fungi of the genus Agaricus for the production of a ripening form of a desired polypeptides according to the method of claims 32-36.

1/15

fig -1

Restriction map of the genomic DNA of *hypA* and *hypC*



2/15

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Proefstation voor de Champignoncultuur
(B) STREET: Peelheideweg 1
(C) CITY: Horst
(D) STATE: Limburg
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 5960 AA
(G) TELEPHONE: 31 4764 7575
(H) TELEFAX: 31 4764 1567

(A) NAME: Moleculaire Genetica van Industriële
Microorganismen
(B) STREET: Dreyenlaan 2
(C) CITY: Wageningen
(D) STATE: Gelderland
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6703 HA
(G) TELEPHONE: 31 8370 4692
(H) TELEFAX: 31 8370 4011

(ii) TITLE OF INVENTION: titel

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Agaricus bisporus
(B) STRAIN: Horst#U1

(vii) IMMEDIATE SOURCE:

(B) CLONE: pIM3100

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 45..383
(D) OTHER INFORMATION: /product= "Hydrophobic protein A"
/gene= "HypA"
/standard_name= "Hydrophobin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

SUBSTITUTE SHEET (RULE 26)

3/15

fig - 2

CTCATTCTTA GCTCTTACAA CTCTCTCCAA GCTTTCGTTG CACA ATG ATC TCT CGC	56
Met Ile Ser Arg	
1	
GTC CTT GTC GCT GCT CTC GTC GCT CTC CCC GCT CTT GTT ACT GCA ACT	104
Val Leu Val Ala Ala Leu Val Ala Leu Pro Ala Leu Val Thr Ala Thr	
5 10 15 20	
CCT GCT CCC GGA AAG CCT AAA GCC AGC AGT CAG TGC GAC GTC GGT GAA	152
Pro Ala Pro Gly Lys Pro Lys Ala Ser Ser Gln Cys Asp Val Gly Glu	
25 30 35	
ATC CAT TGC TGC GAC ACT CAG CAG ACT CCC GAC CAC ACC AGC GCC GCC	200
Ile His Cys Cys Asp Thr Gln Gln Thr Pro Asp His Thr Ser Ala Ala	
40 45 50	
GCG TCT GGT TTG CTT GGT GTT CCC ATC AAC CTT GGT GCT TTC CTC GGT	248
Ala Ser Gly Leu Leu Gly Val Pro Ile Asn Leu Gly Ala Phe Leu Gly	
55 60 65	
TTC GAC TGT ACC CCC ATT TCC GTC CTT GGC GTC GGT GGC AAC AAC TGT	296
Phe Asp Cys Thr Pro Ile Ser Val Leu Gly Val Gly Gly Asn Asn Cys	
70 75 80	
GCT GCT CAG CCT GTC TGC TGC ACA GGA AAT CAA TTC ACC GCA TTG ATT	344
Ala Ala Gln Pro Val Cys Cys Thr Gly Asn Gln Phe Thr Ala Leu Ile	
85 90 95 100	
AAC GCT CTT GAC TGC TCT CCT GTC AAT GTC AAC CTC	380
Asn Ala Leu Asp Cys Ser Pro Val Asn Val Asn Leu	
105 110	
TAGGGAGGCT GATAGCTTCC TTCGTTGACT CGAGATTACT CATAGGGCTT GGAAGAACAG	440
TTCTAGTTTT ATCGTCAGTA TTCTCATGAA GAGATGTTTT CTTGTTGAC TCGGAATGTG	500
CGTTTCAATA TACTGCCTTG GTATTATCTT TCGTT	535

4/15

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4136 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Agaricus bisporus
- (B) STRAIN: Horst #39

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pIM3104

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION: 1..558

(ix) FEATURE:

- (A) NAME/KEY: TATA_signal
- (B) LOCATION: 454..460

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(559..612, 666..729, 786..955, 1013..1060)
- (D) OTHER INFORMATION: /product= "Hydrophobic protein A"
/gene= "HypA"
/standard_name= "Hydrophobin"

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 613..665
/number= 1

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 730..784
/number= 2

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 956..1012
/number= 3

(ix) FEATURE:

- (A) NAME/KEY: misc_RNA
- (B) LOCATION: 3432..3944
- (D) OTHER INFORMATION: /product= "Putative Hydrophobic protein C"
/gene= "HypC"
/standard_name= "Hydrophobin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

SUBSTITUTE SHEET (RULE 26)

5/15

fig - 3.1

AGATCTGAGA CTCAAGATGA CGTCCACTGG CGCACGCGAC TAATGGGACC AGACGTTGCA	60
TTTGGGTTTC AATCGCTCCC TGTCGACGAT GACCCATCGG AAAGCCACTT GCTGGGTCCA	120
CTAGTGGCTT GTTCACTATG ACGCATTGTG CACTAGTGGG TGGGAGGACG AAGGACGGAT	180
CTGATTTGAA TCAAAAGGCC CTCAATGGCT CTGTAGTGAC GGTGCTTTGT TAAAGTGGTC	240
GAGATTGGAG CGAAGGCCTC GGTCCGCCGA GCCAGAAGGG AAGGCAAAGT TCAAATGCTG	300
TCAGACGTAA GCCAGCCGTG GGAGGCCAAA GCTTCTCGAC CTCGATCTTT TTTCTATCCG	360
TGCTTCTTTC TCGTCTCTCC CATCGCTCCA CGGCCGTCT CTTCATGAGG TCTCCTTGGA	420
CGTGATTAAT CTCTATTTGG ATGTCAGCTC TGGTATATAA GATGGTCGCG ACGCACCCAG	480
ACTCTTCAGC TCGCTCTCGT TCTTCGTTTC GCGTCTCATT CTTAGCTCTT ACAACTCTCT	540
CCAAGCTTTC GTTGACAAAT GATCTCTCGC GTCCTTGTCG CTGCTCTCGT CGCTCTCCCC	600
GCTCTTGTTA CTGTGAGTTG ATTGCATGCG CCTCGTTGGC TGAAAAATCT CACTCTCTTT	660
TGCAGGCAAC TCCTGCTCCC GGAAAGCCTA AAGCCAGCAG TCAGTGGGAC GTCGGTGAAA	720
TCCATTGCTG TAAGTATCTC CCATGACACC GCGCACTCC ATATCCTCAG CGACTTCTTT	780
TTTAGGCGAC ACTCAGCAGA CTCCGACCA CACCAGCGCC GCCGCGCTG GTTTGCTTGG	840
TGTTCCCATC AACCTTGGTG CTTTCCTCGG TTTGACTGT ACCCCCATTT CCGTCCCTGG	900
CGTCGGTGGC AACAACTGTG CTGCTCAGCC TGTCTGCTGC ACAGGAAATC AATTCGTAAG	960
TTAGAGCATT TGGCCTTTGA CTTGATTTTA ACACTATTTT TTCTCTCTTC AGACCGCATT	1020
GATTAACGCT CTTGACTGCT CTCCTGTCAA TGTCAACCTC TAGGGAGGCT GATAGCTTCC	1080
TTCGTTGACT CGAGATTACT CATAGGGCTT GGAAGAACAG TTCTAGTTTT ATCGTCAGTA	1140
TTCTCATGAA GAGATGTTTT CTTGTTTCGAC TCGGAATGTG CGTTTCAATA TACTGCCTTG	1200
GTATTATCTT TCGTTTTTCC TGTGTGCATA CTTGAGTTTT TGCTTGAACT TGGAGTCGTG	1260
TAATAGACGA TTTCGAGTAA GAAACTACCA GATACAGGTA CAGGCGACCT CGTTGCCGAC	1320
ATATAAAATA AGATTCCGCG AGTTTTCTTT GTGGGCTCGT TTATTCTTAC GGACAGTCGT	1380
CCAGTCAGCC CCAAGCCCAA GATATCCTTG GACCACCACT GCGAGTTGCG GCAAATTGAA	1440
AATTAATAA TTTGGGGCTT GTAGTTTACA TATAACAATA GTATTTCTCA CATTTTGCAT	1500
TCGGAGATGA AAAATGTGGA GTTCAAGATG ATGGCCCTAT ACTTTCTAAA AATCAATCTA	1560
AACTGTAATT TCAACCCTTG GCTACTGATC ACTTTTTCGT CGTGAGCCCG ATAGTCGCTG	1620
GCCAACCAAC ATTAAAGGAT GGGGATTGAG GATCTAGGGT TGTAGGGCCC GAGATCCCCA	1680

SUBSTITUTE SHEET (RULE 26)

6/15
fig - 3.2

AGTGGTGAGA	TCGAAGCTGG	AACGCAGTCG	GGTCACCCCA	AAAGCGTCAA	CTTCGACCCC	1740
GGTGTGGCT	AGCCTGACTT	CCAGTCTAAG	TAGGGTTAGG	GTGGGTGGAA	AAAATAAAAA	1800
TAGTGGTGCG	GCACTGCGTA	CGACCGCACC	AGTAGAGGTA	GTAGTAGTAG	AATAAGTTAT	1860
AAACCGTGAC	GTGACAGTTA	TATATAGATG	TCGCTCACAC	ACACGGACAT	CACTCCGAAA	1920
ACTCAGACCG	CGCTCATTCC	TCGCGGGTTC	TTCCACAAAG	GTAATATTTC	AAGCTCATGG	1980
CCCCTAGCGT	TTGCTCCTAA	AAGACATGCA	AGACAGAGTT	GCGAAATTGC	TAAAAGCCTA	2040
CTTGCGTGGC	ACTCCATTTA	TGGCCTGTGG	TTCAAGAGGA	GTCAGATTTC	CCGCTGGTTT	2100
GTCGCGGCGT	GGGATATCCT	CACCCTGAAC	TAAACCAAAA	GCGAGAATTG	GCAAACGAGC	2160
GGCGACACAA	CCTGACACAA	CGGCGACAAG	TTGTCTATCA	ATGATCATCT	TGGCCATCAA	2220
CTGGCATCGT	ATAAACTGAT	GCTCTCGGCA	TCCCCAATTT	CATAAGCCGA	CAAAAGCTGT	2280
GCGCATTACA	AAAAACAAAG	TAAGTAATAT	AACAGAAAAA	CTCGATCAGC	TCACTATATA	2340
TGCTATAATT	AGGTGCTTGT	TGCCAGCGCC	AGAAGAATTT	ACTTCTGGAA	TTCCTAAGAG	2400
GCTGACTAGA	AATGTTACAA	CAGGGAGGCT	AGACTTCGGA	AATGCGGATG	AATTGTGCGAC	2460
TGGAGTACAC	GAGTCCACGA	CGCAAACTC	AAGGGAAGTC	GAGTAGAAAG	TTGATCTGCG	2520
AGATCCATTT	TTAACTCGCA	TCGACTACAC	ATCAGCCTGG	TGACATCCAT	TGCCATGAAG	2580
CTGTTGTGAT	AAACTGGTGG	TCATATGCAG	CCGACACATT	CAAAGCTGCA	AGCTGGCCGC	2640
GCAACCGTCC	CCGAGGCTCA	AAGAGGCTCA	AAGCCATTGG	AATTTGGCAA	CGACTGAACC	2700
ATATACGGAG	GGTATTCTC	CTCCACACAT	GACCTCCAAT	GGATTGGCGA	ATCCGACGCC	2760
CCCCGATCCC	CCGACCACCC	AACCACCCGA	CTGGATTGTG	AACAATAGGG	TTTTTCTTCG	2820
ACCCCTGCTT	GGCCTCTAGA	CTAAGCAGAG	TCCCACACGT	CCACTTTGTC	TTACTTTTTTA	2880
GCGAACAAAT	CACGCTTATA	GTCGACCAAA	GAACCTAAGC	CAGTACTAAG	GGGCTAGTCG	2940
TCAGGTGTCC	GAATGTCATT	GACTCCTCCA	AAAAAATACG	CGTTGCGAAT	CTGATACCTC	3000
TCTTTTCGGA	CCTGTCTAGT	TAGGGGACAC	TTTCGCAAGG	TTGAAACATT	GATGTTTCGAT	3060
TTAATTACCA	TAAAAGGATT	CTGCCGAATA	TTGCCATTCA	GCCAAGTGCC	AACACCTTTC	3120
GGCGCGACCC	AAGAGGGAAA	GCAAGATTCC	AAATGCTGTT	AGACATGTAC	AAAAGCTAAT	3180
ACGTGGTGGC	CAAAAGCTCC	TCAACCCCGG	ATCTCGTTTC	CGATCCGTGC	TCCTATCTGG	3240
TCTTTCCAAT	TACTACTCCA	CGGCTTGTCT	CCTCATGAGG	TTTCCTTGGA	CTTAATAAAT	3300
CTCTATTTGG	ATGTCAGCTC	TGGTTTATAA	GGCGGTCGCG	ACGCACTCAG	ACTCTTCAGC	3360
TCGCTCTCGT	CTTTCGTTGG	CACCTCTCATT	CTTAGTTCTT	ACAACTCCCC	CCAAACTTTC	3420

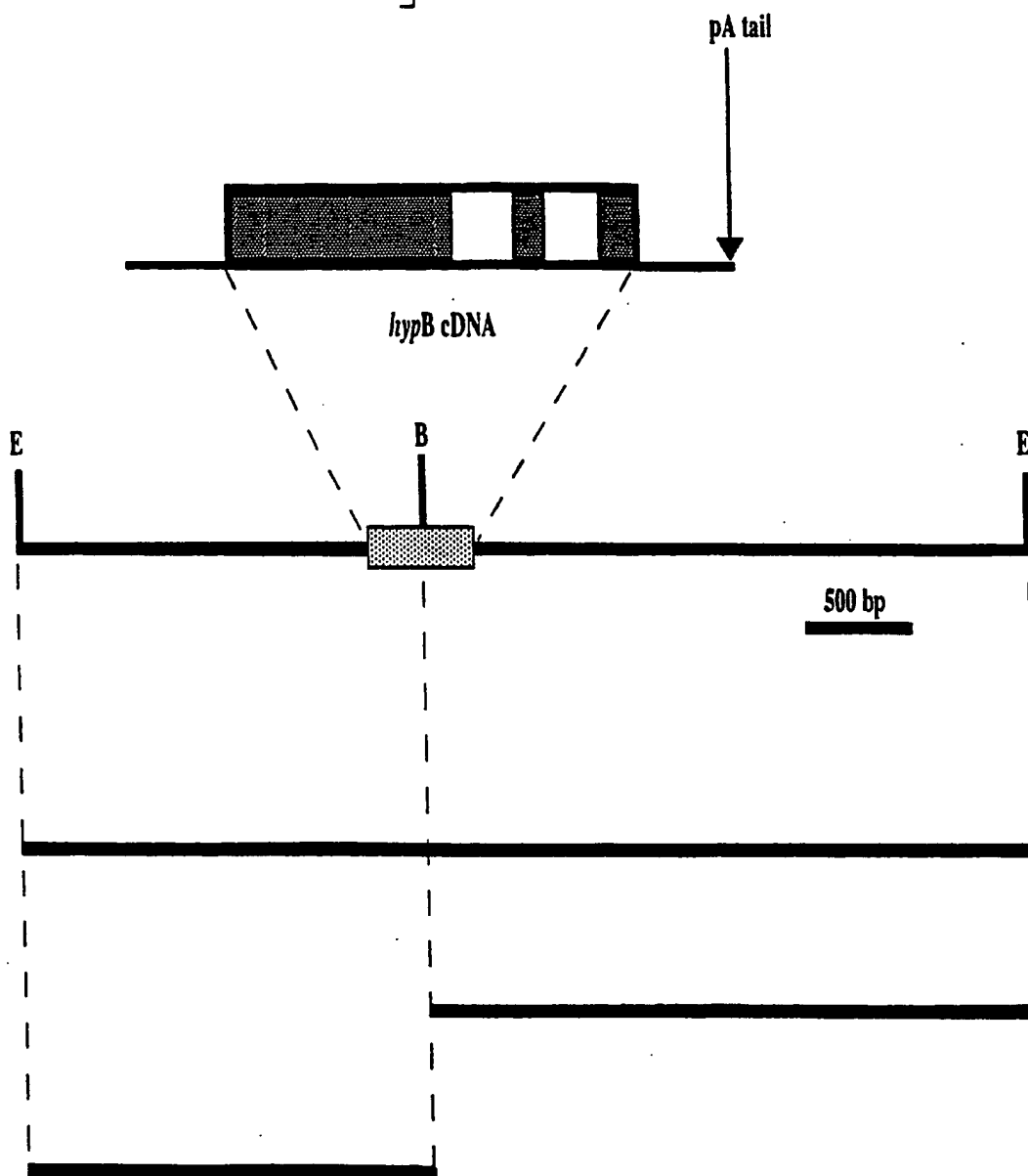
7/15

fig - 3.3

GTTTTACACA ATGTTCTCTC GCGTCCTCGT CGCAGCTCTT GTTGCTCTCC CGGTTCTCGT	3480
TTCCGTGAGT TGACTACCCT CATTGACTGA AATCATTTAC TCACCCTCTT TCGTAGGCAA	3540
GTCCCACTCT GGGGGATACC CTGACTCCAC CACCGTGGTT AGTCAATGCA ACGTCGGTGA	3600
ACTCCACTGC TGTAAGTCTA TTCCATGACA TCTATGTACT CCATATCCTC AGTGACTTCT	3660
TCCAGGCAAC ACTCAGCAAA CCCCCGACCA CACCAACGCC GCCGGCGGTC TGCTTGGTGC	3720
TGCCGCCAAC GTCGGCGCCC TCCTCGGTTT CGACTGTACC CCCATTTCCG TCATTGGCAT	3780
CGGTGGCAAC AACTGTGCTG CTCAGCCCGT CTGCTGTGAA GCCAACGAAT TCGTAAGTTA	3840
GAGCTTCTGG TCTTTGACTT AAAATCTAAT ACTATTCTCT CTCTCTCTCC CTTCAGACCG	3900
GGTTGATTAA TGCTCTTAGC TGCTCTCCCA TCAATGTCAA CCTTTAGGGA GGCTGATGGT	3960
TTCTTCGTC GACTCGAGAT TACTCGGAAG GCTTGGAAGG ACAATTCTAG TTTTATCATC	4020
AGTATTCTCG TGAAACAGAT TTTTATCTTG TTCTTATGAC GGACTCGGGA TATCTCTTTC	4080
AATATACCGC CTTGGTTTAT CTTTCGTTTT TAATTTTGTG TGCACACTTG AGATCT	4136

8/15

fig - 4



9/15

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 481 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Agaricus bisporus
 - (B) STRAIN: Horst#U1
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: pIM3101
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 64..423
 - (D) OTHER INFORMATION: /product= "Hydrophobic protein B"
/gene= "HypB"
/standard_name= "Hydrophobin"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

10/15

fig - 5

CTCATCGCAC TCATTTCACCT TTCCTCAGTT GTCTTCATAT CTTAATCACC TGAGCAAAAA	60
AAA ATG GTC TCA ACG TTC ATC ACT GTA GCA AAA ACC CTT CTC GTC GCG	108
Met Val Ser Thr Phe Ile Thr Val Ala Lys Thr Leu Leu Val Ala	
1 5 10 15	
CTC CTC TTC GTC AAT ATC AAT ATC GTC GTT GGT ACT GCA ACT ACC GGA	156
Leu Leu Phe Val Asn Ile Asn Ile Val Val Gly Thr Ala Thr Thr Gly	
20 25 30	
AAA CAT TGT AGC ACC GGT CCT ATA GAG TGT TGT AAA CAA GTC ATG GAT	204
Lys His Cys Ser Thr Gly Pro Ile Glu Cys Cys Lys Gln Val Met Asp	
35 40 45	
TCT AAA AGT CCT CAA GCT ACG GAG TTG TTG ACG AAA AAT GGC CTT GGA	252
Ser Lys Ser Pro Gln Ala Thr Glu Leu Leu Thr Lys Asn Gly Leu Gly	
50 55 60	
CTG GGT GTA CTT GCT GGA GTG AAA GGT TTG GTT GGA GCG AAT TGT AGT	300
Leu Gly Val Leu Ala Gly Val Lys Gly Leu Val Gly Ala Asn Cys Ser	
65 70 75	
CCT ATC ACG GCA ATT GGT ATT GGA TCC GGA AGC CAA TGT TCT GGA CAG	348
Pro Ile Thr Ala Ile Gly Ile Gly Ser Gly Ser Gln Cys Ser Gly Gln	
80 85 90 95	
ACC GTT TGT TGC CAA AAT AAT AAT TTC AAC GGT GTT GTC GCT ATT GGT	396
Thr Val Cys Cys Gln Asn Asn Asn Phe Asn Gly Val Val Ala Ile Gly	
100 105 110	
TGT ACT CCC ATT AAT GCC AAT GTG	420
Cys Thr Pro Ile Asn Ala Asn Val	
115	
TAATGAGAGG TTTCCTGTCG TTGATATACG TTTGGCTTGA ATAATATGTC TACTTGAAGC	480
G	481

11/15

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 831 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Agaricus bisporus
- (B) STRAIN: Horst #39

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pIM3106

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION: 1..164

(ix) FEATURE:

- (A) NAME/KEY: TATA signal
- (B) LOCATION: 40..46

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(165..339, 519..556, 628..674)
- (D) OTHER INFORMATION: /product= "Hydrophobic protein B"
/gene= "hypB"
/standard_name= "Hydrophobin"

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 440..518
/number= 1

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 557..627
number= 2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

12/15

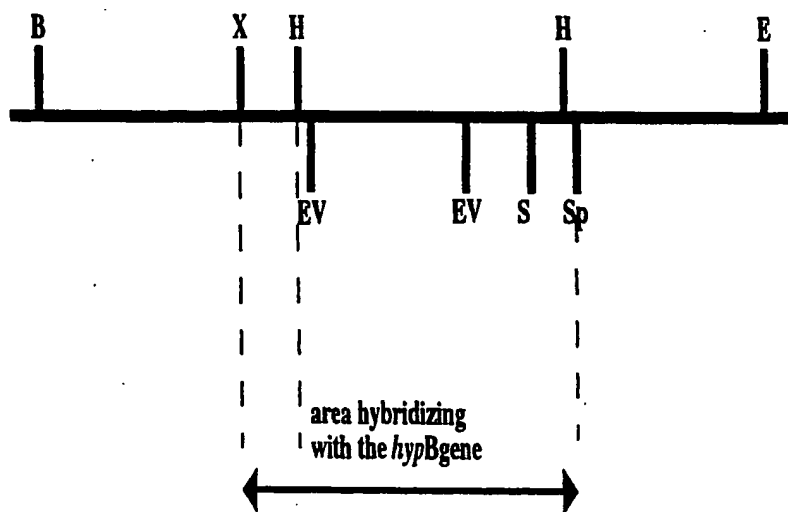
fig - 6

CTACGACCCG TAATACATCG TTAGTGTCTC ACTAACTAGA TATAAAGAGA AATCTCGAAG	60
GATGAAATTA CAATTACACG AAGTGTCAAT CAAAGCCCCA GTCATCGCA CTCATTTAC	120
TTTCCTCACT TGTCTTCATA TCTTAATCAC CTGAGCAAAA AAAAATGGTC TCAACGTTCA	180
TCACTGTAGC AAAAACCCTT CTCGTCGCGC TCCTCTTCGT CAATATCAAT ATCGTCGTTG	240
GTA CTGCAAC TACCGGAAAA CATTGTAGCA CCGGTCCTAT AGAGTGTGT AAACAAGTCA	300
TGGATTCTAA AAGTCCTCAA GCTACGGAGT TGTTGACGAA AAATGGCCTT GGACTGGGTG	360
TACTTGCTGG AGTGAAAGGT TTGGTTGGAG CGAATTGTAG TCCTATCACG GCAATTGGTA	420
TTGGATCCGG AAGCCAATGG TACGTTTTGG AATGGATTGA TTTGTTTCTG TGATTACGCT	480
GGATGTTGAT TAGCTCTTCC CCCCCCTTTT TTTTITAGTT CTGGACAGAC CGTTTGTTGC	540
CAAAATAATA ATTTCACTGA GACTGACCAC CCTCTCTCTC TTTCTGAGCT TTTCCGCGGT	600
TCGAATACTG ATTTATCCTC TTTTCAGACG GTGTTGTCGC TATTGGTTGT ACTCCATTA	660
ATGCCAATGT GTAATGAGAG GTTCCTGTC GTTGATATAC GTTTGGCTTG AATAATATGT	720
CTACTTGAAG CGAGCTAAGT TAAACGGGAC TCGTCGTCCT CAAGTCCGGA TCATGAATAG	780
GTAAAAATGT GAGTAATAAG AAATAAGGGA GAGGAAACCG AATGGTCCGA T	831

13/15

fig - 7

500 bp



14/15

fig - 8

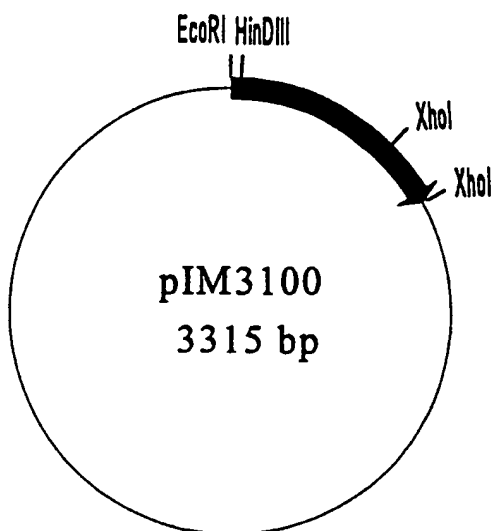


fig - 9

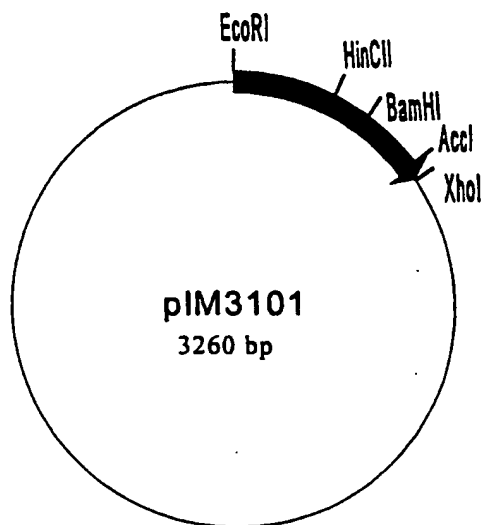


fig - 10

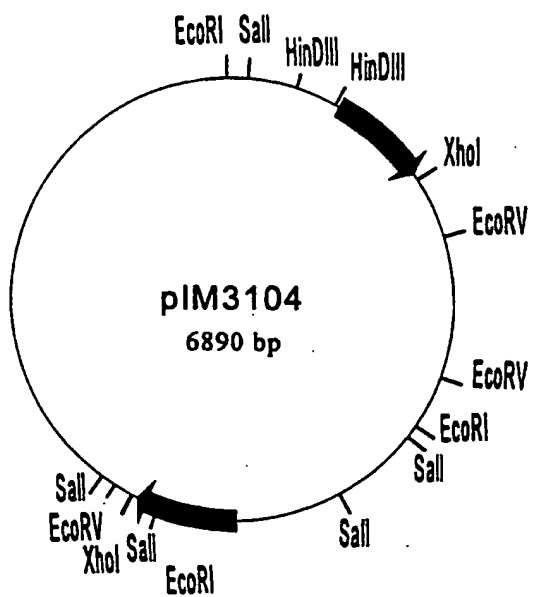
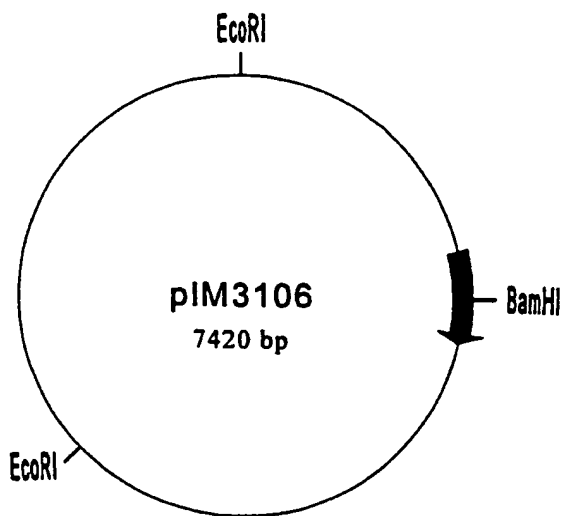
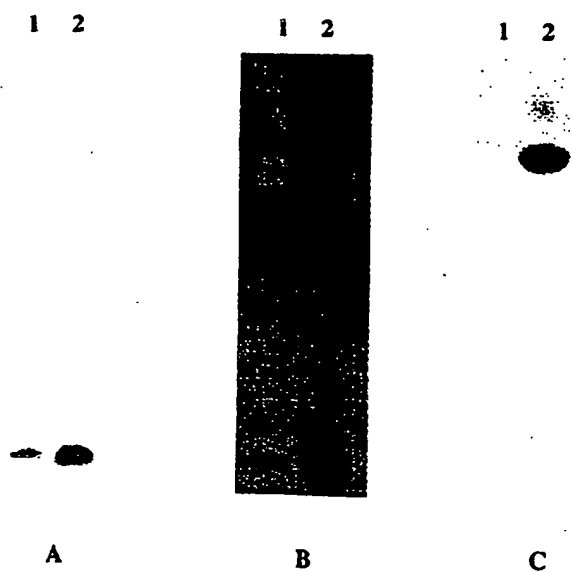


fig - 11



15/15

fig-12



INTERNATIONAL SEARCH REPORT

Inte Application No
PCT/NL 96/00234

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/31 C12N15/80 C07K14/375 C12N1/15 C12N1/21 A23L1/00 A01N63/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARCH MICROBIOL, 159 (4). 1993. 330-335., XP000601375	1-4
Y	DE VRIES O M H ET AL: "INSOLUBLE HYDROPHOBIN COMPLEXES IN THE WALLS OF SCHIZOPHYLLUM-COMMUNE AND OTHER FILAMENTOUS FUNGI" see the whole document	5-20
X	--- PROC NATL SCI COUNC REPUB CHINA PART B LIFE SCI, 16 (3). 1992. 134-143., XP000601379	1-4
Y	WESSELS J G H: "PROTEIN EXCRETION AND MORPHOGENESIS IN FUNGI" see the whole document ---	5-20
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*&* document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search	Date of mailing of the international search report	
20 September 1996	15. 11. 96	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Gurdjian, D	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 96/00234

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DNA SEQUENCE (1992), 3(3), 131-41 , XP000601186 EBERLE, JUERGEN ET AL: "Neurospora crassa blue-light-inducible gene bli-7 encodes a short hydrophobic protein" see the whole document ---	5-20
A	WO,A,95 02691 (INSTITUUT VOOR AGROTECHNOLOGIS ;C N C COOPERATIVE NL CHAMPIGN (NL) 26 January 1995 see claims 1-38 ---	30-37
A	NEW PHYTOLOGIST, 1993, XP000601366 WESSELS JGH: "WALL GROWTH, PROTEIN EXCRETION AND MORPHOGENESIS IN FUNGI" see page 407, left-hand column, paragraph 2 - page 410, right-hand column, paragraph 1 ---	1-20
A	ANNU. REV. PHYTOPATHOL., vol. 32, 1994, pages 413-437, XP000601369 WESSELS J.G.H. ET AL.: "Developmental regulation of fungal cell wall formation" cited in the application see page 423, paragraph 2 - page 430, paragraph 2 -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

Information on patent family members

Ln

Application No

PCT/NL 96/00234